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Medical Lib

THE JOURNAL OF GENERAL PHYSIOLOGY

Founded by Jacques Loeb

EDITORS

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W. J. V. OSTERHOUT

VOLUME VII, No. 1 SEPTEMBER 20, 1924

PUBLISHED BIMONTHLY

AT MOUNT ROYAL AND GUILFORD AVENUES, BALTIMORE, MD.

BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

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MEMBRANE EQUILIBRIA AND THE ELECTRIC CHARGE OF RED BLOOD CELLS.

By CALVIN B. COULTER.*

(From the Pathological Laboratory, Brooklyn Hospital, and the Department of Bacteriology, Columbia University.)

(Accepted for publication, May 6, 1924.)

The present work is an investigation into the electric charge of red blood cells from the point of view of the Donnan membrane equilibrium. It deals with the existence of an equilibrium state in the distribution of ions between the cell and a medium of simple composition, and the potential difference at the cell membrane arising from unequal distribution of H and Cl ions on opposite sides of the membrane; and the relation of this P.D. to that calculated from the rate of migration in cataphoresis.

The idea that a "concentration chain" might produce an electric P.D. of biological significance was considered by Höber[1] in connection with the first published observations on the cataphoresis of red blood cells. Ostwald[2] had pointed out the effect of a semipermeable membrane in limiting diffusion, but it was not until the development of the theory of membrane equilibrium by Donnan[3] that it became possible to deal with quantitative data. Warburg[4] and Van Slyke[5] have applied this theory to the red cell plasma system in papers which have appeared while the present work was in progress, and which deal with ion concentrations that apply to the physiological rôle of the

*Van Cott Fellow in Pathology.

[1] Höber, R., *Arch. ges. Physiol.*, 1904, ci, 607.

[2] Ostwald, W., *Z. physik. Chem.*, 1890, vi, 71.

[3] An interpretation of the theories of Donnan is given by Lewis, W. C. McC., A system of physical chemistry, London and New York, 3rd edition, 1920, ii, and by Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 2nd edition, 1922.

[4] Warburg, E. J., *Biochem. J.*, 1922, xvi, 153.

[5] Van Slyke, D. D., Wu, H., and McLean, F. C., *J. Biol. Chem.*, 1923, lvi, 765.

cells. The numerous papers of Jacques Loeb appearing in the *Journal of General Physiology* on the application of the Donnan equilibrium to protein systems present the background of the present study.

Observations reported in a previous paper[6] had shown difference between the pH of the interior of red cells and that of the medium, so large that it seemed impossible to explain them by the Donnan equilibrium. It was found further that fresh red cells, when washed and suspended in saccharose solution showed a higher charge in cataphoresis than similarly washed cells which had been preserved 24 hours or longer in saccharose solution. This made it appear doubtful that the ionization of the cell protein was the sole source of the charge and it seemed possible that the cells owed their charge in part at least to a simple diffusion potential, due to diffusion outward of salt contained within the cell. Such a charge might be high when the cells were first placed in an electrolyte-free medium, and would fall to zero as soon as diffusion had brought the salts to the same concentration on both sides of the cell membrane. In view of the observed changes in pH of the medium, the most important salt in this connection appeared to be the phosphates of the cell. Accordingly the amount of phosphate which had diffused out of the cells was determined by the method of Tisdall[7] in the supernatant fluid from equal amounts (4 cc.) of washed sheep cells which had been allowed to stand for varying lengths of time in like quantities (40 cc.) of isotonic saccharose solution at about 10°C.

One experiment, which was repeated numerous times with the same general result, is given in Table I.

The amounts are so small that their exact determination is at the limit of accuracy of the method, but the relative error between determinations is probably small. It is apparent that the greater part of the total PO_4 which diffuses from the cells in 24 hours does so within the first 5 minutes or less. The subsequent rate of diffusion is so low that the condition approximates an equilibrium in the distribution of PO_4 ions between the cell and the medium. A potential due to diffusion of molecular phosphate must therefore be infinitesimal.

[6] Coulter, C. B., *J. Gen. Physiol.*, 1921–22, iv, 403.
[7] Tisdall, F. F., *J. Biol. Chem.*, 1922, l, 329.

Measurements of the H concentration in the medium and in the interior of cells give evidence of the attainment of a like condition of equilibrium in the distribution of this ion. This is true both when the medium is originally electrolyte-free and when it contains salts, although the H distribution is markedly affected by the presence of salts.

Cells from fresh defibrinated sheep blood were washed twice with five volumes of isotonic NaCl solution and four times with similar volumes of saccharose solution; 4 cc. of strongly sedimented cells were added in the majority of experiments to 36 cc. of isotonic solution, in stoppered tubes. These were placed in the refrigerator at 5°–15°C. After varying intervals the supernatant fluid was separated by the centrifuge and removed as completely as possible without disturbing the cell sediment. The latter was then dissolved in 20 cc. of dis-

TABLE I.

Mg. $\times$ 10^{-3} *Phosphorus Lost by 4 Cc. of Cells.*

5 min.	1 hr.	3 hrs.	24 hrs.
9.0	9.5	10.4	11.2

tilled water. The water used for all solutions was distilled, with occasional exceptions, the same day it was used. The pH measurements were made with freshly palladinized electrodes in vessels similar to that of Clark,[8] using a separate electrode and vessel for each specimen of fluid. The vessels were not agitated. The battery of electrodes was placed in the refrigerator for 12 to 18 hours, removed one at a time, hydrogen admitted to restore atmospheric pressure, and the E.M.F. read 10 minutes after removal from the refrigerator. The temperature of the solution was taken immediately after the E.M.F. was read. The electrodes were allowed barely to touch the surface of the solutions; those containing dissolved cells showed, after the prolonged stay in the refrigerator, a narrow zone, at the surface, of completely reduced hemoglobin. The usual technique was em-

[8] Clark, W. M., The determination of hydrogen ions, Baltimore, 2nd edition, 1922.

ployed by changing the solution to maintain approximately the original concentration of CO_2 of the solution. Measurements by this method were more consistent than those in which electrode equilibrium was sought by rocking the vessel; the solutions containing much hemoglobin gave considerably higher pH values which could be reproduced within 0.01 pH. Control measurements in cell solution diluted 1:10, 1:5, and 1:1, the last made by freezing and thawing the cell sediment, gave in one experiment the following values: 1:10, pH 7.277; 1:5, pH 7.273; 1:1, pH 7.253. In several other experiments the 1:10 dilution gave values from 0.03 to 0.06 pH higher than the

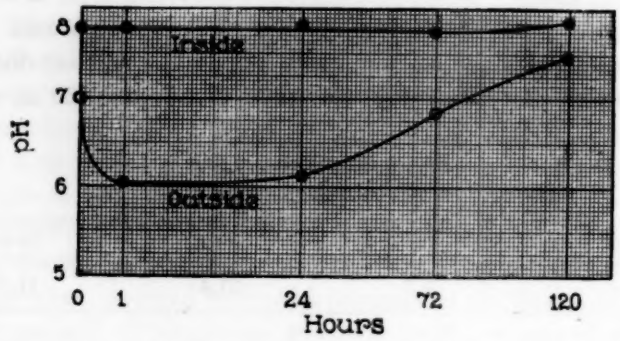

FIG. 1. Reactions of medium and cells suspended in 250 cc. of saccharose solution without salt.

undiluted cell fluid. The change in pH caused by reduction of the oxyhemoglobin to reduced hemoglobin by the hydrogen electrode has been considered too small to require correction for the purpose of this investigation. Frequent colorimetric controls were made of the pH of the outside fluids. Regardless of the method or technique employed for pH measurement, the relations between inside and outside pH were always of the same nature as those shown in the curves.

The buffer value of the cell interior shown by the figures for the effect of dilution in pH is thus very high, and we feel justified in concluding that the pH of the cell has not been altered to a significant degree by hydrolysis, on dissolving the cells in water. The pH of the 1:5 cell solution has been assumed to represent the actual pH within the cell.

In Fig. 1 are given the pH values of the inside (or 20 per cent) cell solution and of the outside solution (or suspending fluid) in an experiment in which 4 cc. of cells were suspended in 250 cc. of pure saccharose solution. The large volume of solution was used to keep the concentration of electrolyte, resulting from simple diffusion from the cells, as low as possible. Under these conditions the reaction which is reached in the outside fluid within the first hour persists without significant change for 24 hours. Other experiments show that this reaction is reached within a few minutes after adding the cells to the solution.

Careful attention has been paid to the apparent increase in acidity of the outside fluid, which reached in the majority of cases about pH 6, but in numerous experiments became as acid as pH 5.6 to 5.8. Hydrogen electrode measurements in solutions of so low conductivity and buffer value as these are difficult and uncertain; nevertheless saccharose solutions made with freshly distilled water gave repeatedly pH values of 6.8 to 7.0, and there could be no doubt of an actual increase in acidity of the solution after contact with the cells. It was given by both electrometric and colorimetric methods, and has been confirmed by Eggerth.[9] It was found that it was not due to a diffusion of CO_2 from the cells. Since it represents a movement of H from a region of low concentration (the cell interior) to one of high, with respect to this ion, it is evident that the H ion, in spite of its great mobility, is not primarily concerned in the process.

The gradual increase in alkalinity of the outside fluid which is observed after 24 hours is due probably to an alteration in the permeability of the cell membrane. If the volume of solution in which the cells are suspended is small, 40 cc. instead of 250 cc., the outside fluid shows the initial increase in acidity described, which is often sufficient to produce immediate agglutination of the cells, and undergoes after 2 to 4 hours a steady rise in alkalinity, reaching as a rule about pH 6.8. With this change in reaction, the stability of the cells increases, and they remain uniformly suspended after gentle agitation of the vessel containing them.

The inside reaction undergoes very slight change, if the cell suspension be kept at 5°C. However, if the cells are stored at room

[9] Eggerth, A. H., personal communication.

temperature, a distinct rise in alkalinity may be observed after a few hours. It seems possible that this is due to the formation of NH_4 within the cell.

If cells which have been washed in saccharose solution be suspended in isotonic solutions of NaCl or $CaCl_2$ the increase in acidity of the outside fluid does not occur, but the pH of this fluid rises and that of the inside falls within a few minutes, so that they approximate each

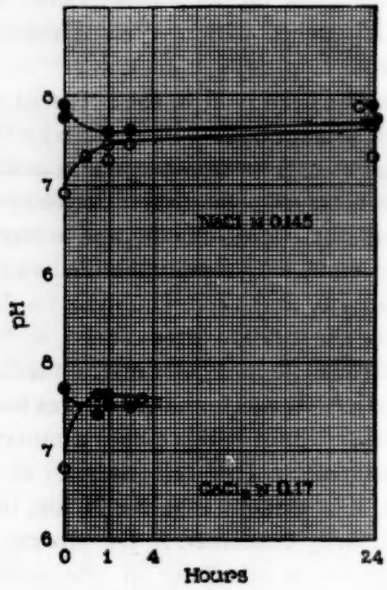

FIG. 2. Reactions of medium and cells suspended in isotonic NaCl and $CaCl_2$ solutions. Black circles, inside pH; white circles, outside pH.

other. This is shown in Fig. 2, which represents two experiments with each of the two salts. In the majority of experiments with NaCl the outside reaction remained slightly more acid than the inside, although in several experiments at the end of 24 hours in the refrigerator the two were identical. With $CaCl_2$ the outside pH rises higher and the inside pH falls lower than in the case of NaCl, so that the inside reaction is now the more acid. Experiments which for the sake of brevity are not detailed have shown that if the concentration of the salt in the suspending fluid is less than isotonic, the isotonicity

of the solution being maintained by saccharose, the changes in pH are intermediate between those just described and those that occur when the outside fluid contains no salt. The extent of change increases with the concentration of the salt.

The reaction attained in the outside fluid when acid is added is affected in a similar way by the presence of salts. In the experiment recorded in Fig. 3, the salts were present in one-quarter of their isotonic concentration, and the same amount of HCl was added before the addition of cells to each of the solutions, bringing them approximately to pH 3.3. The outside reaction remains more acid in the salt-free

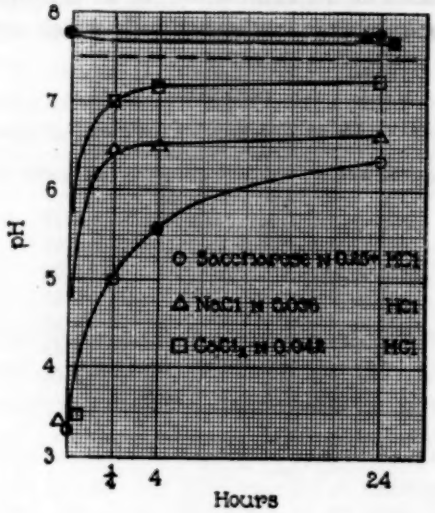

FIG. 3. Reactions of cells and medium to which HCl had been added bringing it to pH 3.3. The salts were present in one-quarter isotonic concentration. Upper curves (black circles) inside pH; lower curves (white circles) outside pH.

solution, and more acid in NaCl than in $CaCl_2$ solution. A close approximation to equilibrium between 4 and 24 hours is evident in the case of the salt solutions. The result is similar when the acid present is H_2CO_3 instead of HCl. Solutions of saccharose and of NaCl, KCl, and $CaCl_2$ were saturated with alveolar air, at about 15°C., bringing them all to the same pH (colorimetric) in each experiment. 4 cc. of packed cells were added to each of the solutions, which

had been covered with a layer of paraffin oil. The concentrations of the salts and the reactions after 80 minutes at 10°C., in two experiments are given in Table II.

The effect of salts is thus the same as when HCl is added. In the more concentrated $CaCl_2$ solution the original relation is reversed and the inside reaction becomes definitely more acid than the outside, as is the case when no acid is added, shown in Fig. 2. Determinations of combined CO_2 in the cells and in the outside fluid gave values which were so irregular as to be without significance.

In order to determine more exactly the distribution of H and Cl ions on the two sides of the cell membrane at equilibrium a separate series of measurements were made with cells which were washed once

TABLE II.

Effect of CO_2 on Reactions of Cells and Medium.

Suspending fluid.	pH outside.	pH inside.
Saccharose	6.27	7.54
KCl N 0.018	6.91	7.23
NaCl N 0.018	6.94	7.27
$CaCl_2$ N 0.021	7.11	7.08
Saccharose	5.65	7.78
NaCl N 0.145	7.43	7.40
$CaCl_2$ N 0.17	7.46	7.31

with saline solution and twice only with saccharose solution, and which were allowed to remain in contact with the outside fluid for 2 hours in the refrigerator. The Cl ratios were determined with AgCl electrodes, using a saturated solution of KCl as a reference solution. Electrodes of C.P. silver were coated with AgCl by electrolysis in an acid solution of N 0.145 NaCl and after thorough washing were allowed to remain in distilled water in the dark for 18 to 48 hours. The electrodes were placed in vessels such as were used for pH measurement. These are provided with a 4-way stop-cock at the lower end so that the two vessels may be connected with a short length of rubber tubing and a salt bridge established by connection with a reservoir of saturated KCl. The contact of the solution to be measured with the salt bridge was made by turning the 4-way cock, the bore of which

contained saturated KCl, until the periphery of this bore was separated from the vessel opening containing the unknown solution by about 2 mm., the stop-cock being greased, and the potential difference between the two electrodes measured with a potentiometer and a sensitive high resistance galvanometer. It was necessary to make the reading very rapidly, before the saturated KCl had opportunity to diffuse into the unknown less concentrated solution. All measurements were made in triplicate, with close agreement between the highest readings on each solution. In order to calculate the pCl of the outside solutions from these E.M.F. values, the E of the saturated KCl electrode was determined by measuring the E.M.F. of the cell: saturated KCl – N 0.1 KCl, and assuming an activity of 0.08 for the N 0.1 KCl solution. From this was obtained the value 0.0286 as the E.M.F. of the saturated KCl electrode against a solution normal with respect to Cl ion. The calculation of pCl from the E.M.F. between the unknown solution and the saturated KCl solution is then at 22° C.

$$\frac{\text{E.M.F. observed} - 0.0286}{58.5} = \text{pCl}$$

The packed cell sediment was suspended in saccharose solution to give a 40 per cent suspension; of this 10 cc. were added to 30 cc. of various concentrations of NaCl and $CaCl_2$ in saccharose solution. After 2 hours stay in the refrigerator, with occasional gentle agitation of the vessels, the supernatant fluid was separated completely after centrifugation and the cells dissolved by adding distilled water to 40 cc., giving a 10 per cent solution. The measurements with H_2 and AgCl electrodes were made on this dilute solution and on the supernatant fluid.

These measurements give the thermodynamic P.D. in millivolts between the two sides of the cell membrane, which is due to the difference in concentration of H or Cl, by subtracting the E.M.F. given by the more concentrated solution, with respect to H or Cl, from the E.M.F. given by the more dilute solution, when measured against a saturated calomel electrode in the case of the H electrode or against the saturated KCl electrode in the case of the Cl electrode measurements. In the latter case the value 58.5 must be subtracted from the

E.M.F. of the cell solution to correct for the 1:10 dilution of the cells. The calculation is as follows:

$Pd\,H_2$ – Saturated calomel chain: E.M.F. inside – E.M.F. outside = H P.D.
AgCl – Saturated KCl chain: E.M.F. outside – (E.M.F. inside – 58.5) = Cl P.D.

The values of H P.D. and Cl P.D. have been plotted as ordinates against the pCl of the outside fluid as abscissæ and are shown as experimental points along the two upper curves in Fig. 4.

If the Donnan equilibrium holds for the distribution of ions between the cell and the medium we should expect these two sets of values to be identical. Experimentally the Cl P.D. is consistently lower than the H P.D. It has been assumed that cells strongly sedimented in the centrifuge are actually concentrated; this was not the case since the cell mass did not show the transparency which develops when intervening fluid is entirely displaced from between the cells. Consequently the actual volume of the cells was less than 4 cc., and the actual Cl P.D. should be greater than that observed. The determinations of the H electrode E.M.F. of the cell solutions may be affected with a considerable experimental error, in spite of the reproducibility of results and are apparently too high. In hydrogen electrode measurements of hemoglobin solutions one is confronted with two sources of error: incomplete reduction, leading to low E.M.F., or the occurrence of chemical changes in the solution, leading possibly to an actual increase in alkalinity. It seemed most important to avoid incomplete reduction and the technique described was devised. It offers the possibility that the unexpectedly high cell pH values are due to the formation of NH_4. However, if H_2 be passed through a fresh hemoglobin solution continuously to a point of complete reduction, NH_4 formation must be insignificant, and the pH approximates but is slightly higher than that given by the technique of prolonged stay in a resting H_2 atmosphere. Furthermore the hemoglobin existed in the cells as oxyhemoglobin but was measured as reduced Hb; correction of the E.M.F. for the increase in alkalinity resulting from reduction would tend to make the H and Cl electrode values approximate, more closely than shown in Fig. 4.

Since the H is concentrated on that side of the cell membrane (outside) on which the Cl is dilute, in all the experiments of Fig. 4,

the P.D. has the same sign of charge in both H and Cl electrode measurements, and in either case the electrode placed in the outside fluid is positive in sign against the electrode which we imagine to be placed within the cell. Further, since the P.D. is determined by the ratio between the concentration inside the cell and that in the outside fluid,

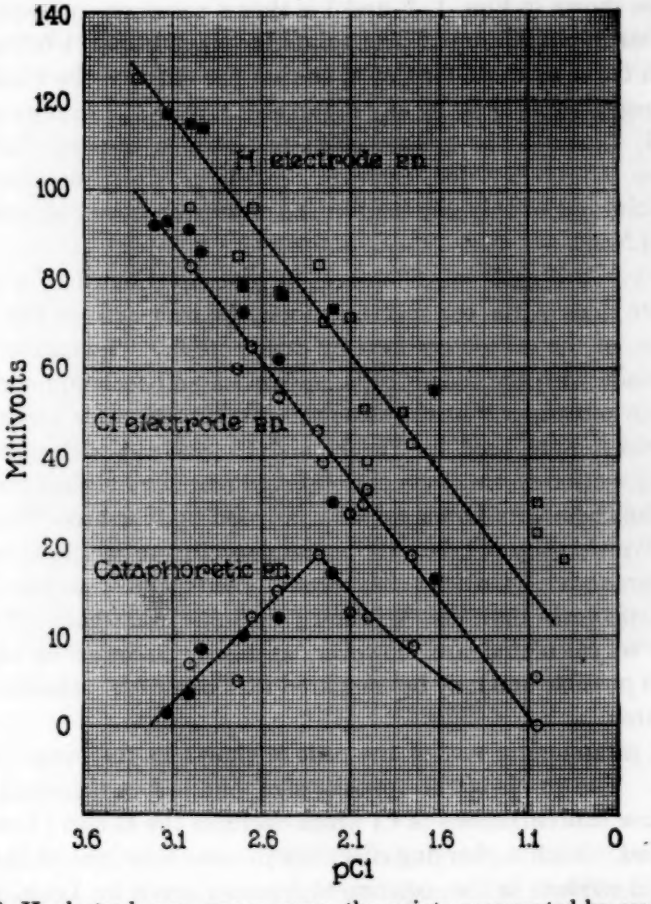

FIG. 4. H_2 electrode P.D., upper curve, the points represented by squares; Cl electrode P.D. middle curve, the points represented by circles. Cataphoretic P.D lower curve. Black squares and circles, $CaCl_2$; white squares and circles, NaCl. The ordinates give P.D. in millivolts, the abscissæ measured pCl values of the outside fluid.

the observed values satisfy the equation $\frac{C_{H_1}}{C_{H_2}} = \frac{C_{Cl_2}}{C_{Cl_1}}$ which is required by the Donnan equilibrium, within the limit of accuracy of the technique.

The effect of the electrolyte upon the reactions of the cell and the medium shown in Figs. 1, 2, and 3 is thus a necessary consequence of the Donnan equilibrium. The points for NaCl in Fig. 4 fall as close to each curve as those for $CaCl_2$, so that the effect of the Ca appears to be negligible as showing any differences from Na. In the curves of Figs. 1, 2, and 3 the greater effect of $CaCl_2$ in causing changes in reaction is probably due, therefore, to the greater concentration of Cl in solutions of this salt than in the approximately equimolar solutions of NaCl.

The P.D. arising from differences in concentration of H and Cl on opposite sides of the cell membrane may be compared in Fig. 4 with the P.D. of the cell determined by cataphoresis. The measurements were made with the macroscopic apparatus and technique described in a previous paper [10] at the same time and on the same specimens of cells which were used for the H and Cl electrode determinations. The calculation of P.D. from the observed rate of movement was made from the Helmholtz-Lamb equation, as used by Northrop [11] assuming a relative viscosity of 1.1 for the isotonic saccharose solution compared with pure water. Criticism has recently been made of such evaluation of cataphoresis data by Winslow[12] and his coauthors. The P.D. values so calculated are, however, as nearly accurate as seems at present possible and may be compared with other P.D. values similarly calculated.

The cataphoretic P.D. of the cells is shown in the lower curve of Fig. 4. The P.D. is of the opposite sign, and is first increased by the same low concentrations of Cl which decrease the H and Cl electrode potential. Such a charging effect low of concentrations of NaCl and $CaCl_2$ is evident in the experimental points given by Loeb, in cata-

[10] Coulter, C. B., *J. Gen. Physiol.*, 1920–21, iii, 309.

[11] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 629.

[12] Winslow, C.-E. A., Falk, I. S., and Caulfield, M. F., *J. Gen. Physiol.*, 1923–24, vi, 177.

phoresis of gelatin-coated and casein particles[13] at reactions on the alkaline side of the isoelectric point, and is very prominent in the cataphoresis of collodion particles.[14] After a maximum is reached, the cataphoretic P.D. of red cells falls with increasing Cl concentration, as does the H and Cl electrode potential, but remains consistently lower.

The marked valency effect of Ca in higher concentrations of $CaCl_2$ than those of Fig. 3 and of La in very low concentration upon the

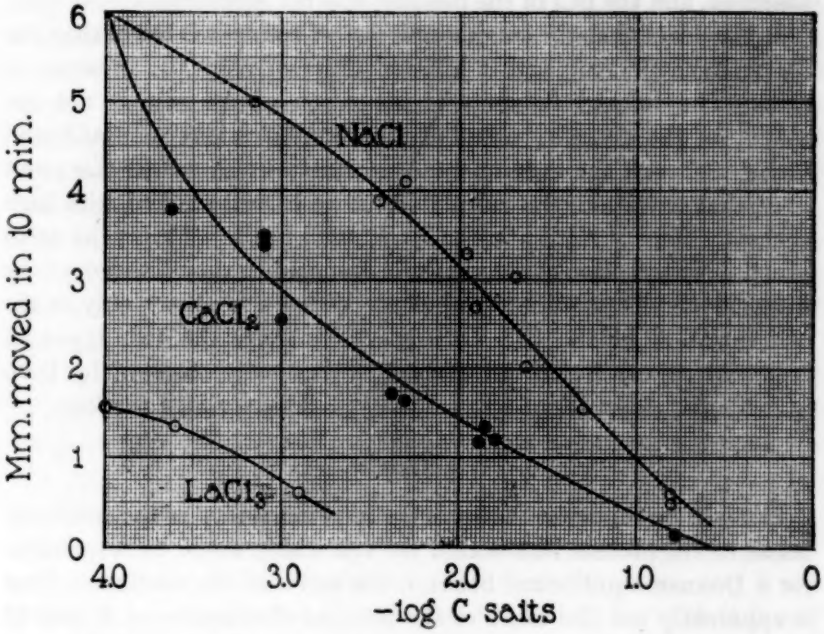

FIG. 5. The rate of movement of red cells under a potential gradient of 3.9 volts per cm. in the presence of NaCl, $CaCl_2$, and $LaCl_3$, at pH 7.

cataphoretic P.D. is shown in Fig. 5, in which the actual rates of movement in mm. per 10 minutes are plotted against the negative log of the molar concentration of the salts calculated from their dilution. The rates shown were extrapolated on a curve drawn between 0 at pH 4.7[10] and the value observed at the pH of the suspending fluid, in

[13] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 395.
[14] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 109.

order to give the rate of movement at pH 7. The curve for extrapolation was drawn conformable to that for the migration of cells in the absence of added salt. The error involved is not of significance as the actual pH values fell between pH 7.1 and 6.2. Only the discharging effect of the salts is shown in Fig. 5 since the left-hand extremity of the curve represents the maximal rate of movement observed in previous experiments at pH 7. The data for this curve were obtained before the charging effects of low concentrations of salts had been observed, and the pCl of the medium was not measured.

It is evident that the cataphoretic P.D. of red cells is lower than the thermodynamic P.D., and is affected differently by the presence of salts. The valency influence of the cation of the salt is not apparent in the charging effect of low concentrations of NaCl and $CaCl_2$, although the experimental inaccuracies may render this point somewhat uncertain; the valency influence is well marked with high concentrations of NaCl and $CaCl_2$ and with $LaCl_3$. This is the usual effect. No influence of the cation is observed in the reduction of the thermodynamic potential; the effect of salts is shown only in the concentration of the Cl. Differences between the two types of potential appear to prevail generally; they were observed by Loeb in extensive observations on various types of suspended particles. [15,16]

DISCUSSION.

It might be supposed that it is the impermeability of the cell membrane to the protein ions within the cell which would be responsible for a Donnan equilibrium between the cell and the medium. That is apparently not the cause of the unequal distribution of H and Cl reported here. At the cell reactions observed, the hemoglobin must exist as K Hb and Na Hb, and if the protein ions were responsible for the Donnan equilibrium, we should expect a membrane hydrolysis to occur, with the development of a more acid reaction within the cell and a more alkaline reaction in the medium. Exactly the opposite change occurs, and we note further that the cation of salts present in the suspending medium has no apparent effect on the

[15] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 505.
[16] Loeb, J., *J. Gen. Physiol.*, 1923–24, vi, 307.

thermodynamic P.D. We are led, therefore, to the conclusion that it is the cations, K and especially Na in the case of sheep blood cells, which are responsible for the equilibrium. Gürber[17] and Doisy and Eaton[18] have shown by direct analyses that K and Na do not pass through the cell membrane, and Van Slyke[5] and his collaborators have considered this fact fundamental in interpreting the electrolyte and water distribution in the blood on the basis of the Donnan equilibrium. The concentration of these cations within the cell is considerably greater than that of protein ions, and Michaelis[3] has considered the probability of potential differences across the cell membrane due to impermeability to inorganic cations. The mechanism of the observed changes in reaction appears to be as follows: in a salt-free medium, the permeating Cl tends to diffuse from the cell because of the great difference in concentration on the two sides of the cell membrane, and since K and Na do not permeate, the Cl carries with it H until the pressure of this ion on the outer side of the membrane equals that of the Cl from the inside. The interior reaction of the cell does not change appreciably because of the high buffer value of the cell.

It is suggested that this is the mechanism of the production of HCl by the cells of the gastric mucosa. To produce the high acidity of the gastric secretion by this mechanism would require the maintenance of a very high Cl concentration within the cell, as well as a means for the removal of the excess of OH ion within the cells.

Increasing concentration of Cl in the outside fluid may be regarded as opposing the diffusion pressure of Cl from the inside, and if the outside C_{Cl} is the greater, Cl will enter the cell taking with it H and increasing the acidity of the cell. This is the result observed with isotonic NaCl and $CaCl_2$ solutions in Fig. 2 and in Table II. If Fig. 2 be compared with Fig. 3, it is seen that a solution of low C_H but high C_{CL} produces a greater increase in acidity within the cell than one of of high C_H but low C_{Cl}. It is well known that the presence of neutral salt makes possible the appreciation of sour taste in a solution of less acidity than in one in which neutral salt is absent.

[17] Gürber, A., *Jahres.-ber. fortschr. Thier-chem.*, 1895, xxv, 165. Quoted by Van Slyke.[5]

[18] Doisy, E. A., and Eaton, E. P., *J. Biol. Chem.*, 1921, xlvii, 377.

If the HPO_4'' ions readily permeate the cell membrane we should expect the ratio $\sqrt{\frac{C_{HPO_4''} \text{ inside}}{C_{HPO_4''} \text{ outside}}}$ at equilibrium to equal $\frac{C_{Cl} \text{ inside}}{C_{Cl} \text{ outside}}$. Although we have found by analyses that the total amount of inorganic phosphate lost by the cell to the medium increases slightly with increasing salt concentration of the medium, the ratio of $\frac{\text{inorganic phosphate inside}}{\text{inorganic phosphate outside}}$ is not that to be expected, since in several experiments it was approximately $\frac{100}{1}$ in cases where the Cl ratio $\frac{\text{inside}}{\text{outside}}$ was very nearly 1. It seems probable, therefore, that the distribution of HPO_4'' is not determined by the same mechanism as that of Cl, and a relative impermeability of the cell membrane to HPO_4'' under the conditions of these experiments, is suggested. The determination of HPO_4'' concentration within the cell is subject to far greater difficulty than is that of Cl. The discrepancy observed may also be due to the fact that only a small proportion of the total phosphate within the cell is in the form of ions.

The impermeability of the cell membrane to inorganic cations is not absolute, since resistant specimens of cells may show after 3 to 5 days suspension in a large volume of saccharose solution such a loss of their electrolyte content, without losing hemoglobin, that they do not undergo hemolysis in distilled water until fifteen times their volume of water is added. When examined microscopically in suspension in five times their volume of distilled water such cells appear wholly normal. The reactions of cell and medium at such a time are those shown in Fig. 1. On the other hand, in solutions of relatively high salt concentration the membrane shows a considerable permeability to hemoglobin, although the Donnan equilibrium applies in this case almost as well as where the membrane is entirely impermeable to hemoglobin.

In the case of gelatin particles studied by Loeb,[19] at acid reactions, at which the non-permeating ion is the gelatin cation, the cataphoretic

[19] Loeb, J., *J. Gen. Physiol.*, 1923–24, vi, 215.

charge is positive in sign, and we should expect that result in the case of red cells, since the non-permeating cations appear to determine the Donnan equilibrium. With both red cells, at the H and Cl concentrations considered here, and acid gelatin particles, the observed thermodynamic potential would be negative in sign if one H (or Cl) electrode were placed within the particle, and one H (or Cl) electrode in the fluid medium; the true membrane potential in this case should be positive in sign, since it is this potential which maintains equilibrium by opposing the thermodynamic or diffusion-pressure potential (Michaelis[3]). This sign of charge corresponds with the observed cataphoretic charge of acid gelatin particles. The cataphoretic charge of red cells in all the experiments of Figs. 4 and 5 is beyond question negative in sign. We should expect it to be positive if both thermodynamic and cataphoretic P.D. have the same origin, as suggested by Wilson.[20] Loeb has considered the valency effect of discharging ions to be strong evidence that the cataphoretic P.D. is determined by the Donnan equilibrium; [16] if this is the case with red blood cells, the cataphoretic P.D. arises conceivably at a different membrane or phase-boundary from that concerned in the thermodynamic P.D. The boundary at which the cataphoretic P.D. is produced in such a case is probably that between the outside of the cell membrane and the medium, since the ionization of the protein of the membrane would be expected to manifest itself here, as it undoubtedly does when the cell assumes a positive charge in reactions more acid than pH 4.7.[10]

SUMMARY.

It has been shown, within the probable limit of error of the methods of measurement employed, that the Donnan equilibrium determines the distribution of H and Cl ions between the cell and the surrounding fluid. This equilibrium is a consequence of the impermeability of the cell membrane to the inorganic cations of the cell. The mechanism responsible for this equilibrium is suggested as that concerned in the secretion of HCl by the cells of the gastric mucosa. If the salt concentration of the medium is low there may result from the Donnan equilibrium a thermodynamic P.D. of considerable magnitude. In

[20] Wilson, J. A., *J. Am. Chem. Soc.*, 1916, xxxviii, 1982.

the presence of low concentrations of electrolytes, this P.D. is to be regarded as positive in sign at reactions of the medium at which the cataphoretic charge of the cell is negative in sign. The explanation of this discrepancy in sign of charge may lie in the existence at an outer phase-boundary of a second Donnan equilibrium the nature of which is determined by the ionization of the protein of the cell membrane.

SOME PHYSIOLOGICAL ACTIONS OF CYANIDES.

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(Accepted for publication, July 18, 1924.)

That cyanides in dilute solutions act as general protoplasmic depressants is well known. In stronger solutions their toxic effects are equally established (Hyman).[1] Inasmuch as these conclusions have been drawn almost exclusively from studies using potassium cyanide, it was thought desirable to see to what extent such effects were due to the alkalinity of the solutions used as well as to their cyanide content.

Potassium cyanide in aqueous solutions is strongly alkaline in reaction due to the manner in which it dissociates.

$$KCN + H_2O \rightleftarrows KOH + HCN$$
$$\uparrow\downarrow$$
$$K^{\cdot} + OH'$$

Hydrocyanic acid, as is well known, is an extremely weak acid, dissociating only to a very slight extent. It is a matter of some importance, therefore, to determine to what extent the physiological properties of a solution of KCN are due to the hydroxyl ions in solution and to what extent they are due to the poorly dissociated HCN molecule. In view of the recent data of Jacobs[2] for carbonic acid and of Beerman[3] for hydrogen sulfide, in which the undissociated molecule has been shown to be of much importance in determining the physiological properties of solutions of these acids, it was thought desirable to study hydrocyanic acid from the same point of view. The experiments herein reported have been carried out with this end in view using various materials: protozoans, tadpoles, and "artificial cells."

[1] Hyman, L. H., *Am. J. Physiol.*, 1919, xlviii, 340.

[2] Jacobs, M. H., *J. Exp. Zool.*, 1912, xii, 519; *Carnegie Institution of Washington, Pub. No. 183*, 1914; *Am. J. Physiol.*, 1920, li, 321; 1920, liii, 457; *J. Gen. Physiol.*, 1922–23, v, 181.

[3] Beerman, H., *J. Exp. Zool.*, 1924, xxxix, (in press).

Experiments on Protozoa.

The effects of CO_2 and of H_2S on various protozoa have been studied by Jacobs[2] and Beerman.[3] These authors found that the order of resistance of various protozoa to these chemicals bears no relation to the order found in the case of other acids which act primarily through their hydrogen ion effects (Collett).[4] Hydrocyanic acid has been found by the author to produce approximately the same effects as carbonic acid and hydrogen sulfide, but with marked differences in velocity for the given species.

Among the forms studied in the present experiments the most extensively used were *Paramecium caudatum*, *Paramecium multimicronucleatum*, *Paramecium aurelia*, *Paramecium bursaria*, *Euplotes patella*, and *Euglena viridis*. Mixed cultures as well as some pure line cultures of these organisms were used. The same calibrated pipette was used throughout, so that the same relative amount of culture was introduced in all experiments. Animals were put in 10 cc. of solutions in Syracuse watch-glasses and kept covered to prevent evaporation. The time taken to kill approximately two-thirds and the time when all were killed was obtained by means of a stop-watch.

Inasmuch as many experiments have been carried out using different concentrations of HCN, and since the results are qualitatively similar, only typical experiments will be cited to show the physiological action of the reagent.

The behavior of protozoa in HCN is strikingly similar to that described by Jacobs[2] and Beerman[3] for CO_2 and H_2S. The animal becomes sluggish, locomotion finally ceases, while the cilia continue beating, eventually stop, and the animal is dead. The bodies of the organisms become slightly swollen, more so in breadth than in length, the nuclei both micro and macro are distinct, while vacuoles from the very beginning of the experiment are engorged and stand out very prominently. No marked cytolysis occurs, except that in *Paramecium bursaria* which is least resistant to the acid the pellicle bursts and an irregularly shaped mass remains after the animal is killed.

[4] Collett, M. E., *J. Exp. Zool.*, 1919, xxix, 443.

In the case of the species of *Paramecia* used the relative toxicity of a solution of HCN of pH 5.6 is as follows: *Paramecium bursaria* is killed in approximately 2 to 3 minutes; *Paramecium aurelia* in 4 to 5 minutes; *Paramecium multimicronucleatum* in 10 to 15 minutes and *Paramecium caudatum* in approximately 1 to 2 hours. *Euplotes patella* is extremely sensitive to HCN, its resistance to the above solution being between that of *Paramecium bursaria* and *Paramecium aurelia*. The time for death ranges from 2 to 3 minutes. *Euglena viridis* is most sensitive to the acid being killed almost instantaneously.

These experiments on protozoa show that different species of these animals have different resistances to HCN. If the effects of this acid are due to hydrogen ions in its solution the same order of resistance ought to be obtained with solutions of other acids, especially when of the same hydrogen ion concentration. The results of Miss Collett[4] and of Jacobs[2] for mineral acids, which have been confirmed by the author, show that the order of resistance of *Paramecia* is *Paramecium bursaria* > *Paramecium caudatum* > *Paramecium aurelia*. The order of resistance to CO_2 (Jacobs[2]) and to H_2S (Beerman[3]) is *Paramecium caudatum* > *Paramecium aurelia* > *Paramecium bursaria*. In the present experiments the order of resistance to HCN is *Paramecium caudatum* > *Paramecium multimicronucleatum* > *Paramecium aurelia* > *Paramecium bursaria*, a condition quite the reverse of that found for mineral acids but similar to that found for CO_2 and H_2S. *Euplotes* is also quite resistant to mineral acids but extremely sensitive to HCN.

Artificial Cell Experiments.

Since Jacobs[2] and Beerman[3] have pointed out that CO_2 and H_2S, in contrast to mineral acids, must act after penetration of the cell, it was thought desirable to test further the possibility that HCN acted in a similar manner. Artificial cells, "the shell vial cell" and the "frog skin cell" as devised and described by Jacobs,[2] were extensively used. The following table, a result of a typical experiment with such "cells," shows that HCN undoubtedly acts in a manner quite similar to the two other acids mentioned.

Frog Skin Cell.

Reagent.	1 per cent HCN	$\frac{N}{100}$ KCN + HCN	$\frac{N}{100}$ NaOH + HCN	$\frac{N}{100}$ KCN + HCL	$\frac{N}{100}$ NaOH + HCL	Tap water.
pH of reagent.	5.5	7.2	7.2	7.2	7.2	7.2
Penetration (intracellular acidity).	++	++	++	+	−	−
Presence of intracellular CN.	++	++	++	+	−	−

This experiment, in which the reagents except HCN were all brought to the same pH value, as indicated in the table, seems to show rather clearly that HCN penetrates the cell and then ionizes, producing an intracellular acidity. That the membranes are not killed by these reagents is easily demonstrated by changing the reagents and substituting mineral acids and other reagents which are known not to penetrate living membranes in the above manner. No changes in intracellular acidity are noted in such check experiments.

The following table, giving the results of a typical experiment with the "frog skin cell" shows that an intracellular acidity can be produced by HCN in an alkaline medium.

Reagent.	HCN	CO_2 saturated H_2O	$\frac{N}{100}$ NaOH + HCN	$\frac{N}{100}$ KCN + HCN	$\frac{N}{1,000}$ KCN	Tap water.
pH of reagent.	6.4	3.8	7.6	7.5	9.5	7.3
Penetration (intracellular acidity).	++	++	++	+	Slight.	−

CO_2 in the concentration used penetrated in about the same relative time as the HCN. No exact quantitative comparisons in the relative rates of penetration of the two reagents have as yet been made. The extreme rapidity of penetration of HCN (in this experiment less than 1 to 2 minutes) must, however, be an important factor in explaining the rapid toxicity of the reagent.

Experiments carried out with bullfrog tadpoles, using the same solutions as in the "artificial cell" experiments, gave the same relative results. The animals were always killed first in those solutions in which the HCN penetrated the "frog skin cells" most quickly.

The results of these experiments show that in many respects HCN acts like CO_2 and H_2S. The experiments on protozoa seem to show quite definitely that HCN, like CO_2 and H_2S, acts from within the cell outward, while the "artificial cell" experiments tend to confirm the view that HCN, as a molecule and not in the ionized state, penetrates the cell. HCN penetrates the cell as a molecule and dissociates within the cell giving rise to H and CN ions. The acid effect within the cell is, of course, due to the H ions.

The importance of controlling the hydrogen ion concentration in experiments with solutions of KCN is thus apparent. This point has been recently indicated in a practical way by Edwards[5] who has shown that unneutralized solutions of KCN produce protoplasmic changes in ameba similar to those caused by solutions of KOH, while neutralized solutions of KCN produce changes of an entirely different nature.

SUMMARY AND CONCLUSIONS.

1. The physiological actions of HCN and its salts appear to be due (*a*) to the ease with which HCN molecules penetrate living cells and then ionizing, exert their influence by means of H ions and CN ions; (*b*) to the weakness of HCN as an acid, which permits at neutrality or at even slight alkalinity the presence of a considerable amount of free HCN molecules in the presence of their salts; (*c*) to specific effects occasioned by its chemical activity.

2. The order of resistance of various protozoans to HCN resembles that of the same protozoa to CO_2 and to H_2S, but is the reverse of their resistance to mineral acids.

3. HCN in acid, neutral, or slightly alkaline media produces intracellular acidity because of the rapid penetration of HCN molecules into the cell.

4. HCN acts specifically on certain species of protozoa, the order of resistance of *Paramecia* being *Paramecium caudatum* > *Paramecium multimicronucleatum* > *Paramecium aurelia* > *Paramecium bursaria*.

I am greatly indebted to the Fumigators Chemical Company, Inc., New York, who have placed at my disposal a generous supply of liquid hydrocyanic acid.

[5] Edwards, J. G., *J. Exp. Zool.*, 1923–24, xxxviii, 1.

THE COMBINATION OF SALTS AND PROTEINS. I.

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(Accepted for publication, July 24, 1924.)

In most of the work on the properties of protein solutions it has been assumed that only the hydrogen and hydroxyl ions react with proteins to form complex ions and that salts are chemically inert. Loeb's experiments[1] on the effect of salts on the Donnan equilibrium show that in dilute protein and salt solutions any reaction between the salt ions and the protein is negligible compared with the reaction between protein and hydrogen and hydroxyl ions. There are indications, however, that other ions may combine with the protein since it has been found by Mellanby, Hardy, Pauli, Robertson, and others[2] that proteins affect the conductivity of salt solutions, and hence that there is presumably a compound formed between the salt and the protein. The marked effect of salts on the precipitation and solubility of proteins (Michaelis) also indicates that some reaction occurs with salts as well as with acids and bases. Kunitz[3] noted slight discrepancies in concentrated salt solutions. If this is actually the case, it would have a marked influence on the distribution of the ions of the salt when added to a system containing diffusible and non-diffusible ions separated by a membrane. It has been shown by Gibbs, and independently and in detail by Donnan, that at equilibrium the product of the activity of any pair of oppositely charged diffusible ions on one side of the membrane must be equal to the prod-

[1] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

[2] For the literature on this subject see Robertson, T. B., The physical chemistry of the proteins, New York, London, Bombay, Calcutta, and Madras, 1918.

It has been found by Hastings in Van Slyke's laboratory that Ca protein salts are only partly dissociated, personal communication from Dr. Hastings.

[3] Kunitz, M., *J. Gen. Physiol.*, 1923–24, vi, 547.

uct of the activity of the same pair on the opposite side of the membrane. Expressed as an equation, this may be written

$$\frac{(A_o^{-n})^{\frac{1}{n}}}{(A_i^{-n})^{\frac{1}{n}}} = \frac{(B_i^{+m})^{\frac{1}{m}}}{(B_o^{+m})^{\frac{1}{m}}} = \cdots$$

in which A_i is the activity of an n-valent negative ion inside the membrane, A_o the activity of the same ion outside the membrane, and B is any positive ion. In dilute solution, the concentrations may be used instead of activities. It was shown by Procter and Wilson[4] that this equation held for the distribution of ions inside and outside of gelatin blocks in equilibrium with a solution of acid. Loeb[1] found that the hydrogen electrode potential between the inside and the outside solution was equal to the calomel electrode potential under the same conditions, and that therefore the ratio of the activities of the hydrogen ion must be equal to the reciprocal of the chlorine ion ratio, as is demanded by the theory. If the ions of a salt do not combine with the protein, the ratio of the total concentration of any cation as determined by analysis should be approximately the same as the ratio of the hydrogen ion activities corrected, of course, for the valence effect; whereas the ratio of the anion concentrations should be equal to the reciprocal of the hydrogen ion ratio. Loeb has shown that this is true for the chloride ion. For other ions, however, this relation has not been investigated in detail. In order to test this point a number of experiments were performed in which various salts were added to suspensions of gelatin particles and the suspension allowed to come to equilibrium at 0°. The solutions were then analyzed and the concentration ratios compared with the hydrogen ion activity ratios as determined with the hydrogen electrode. The results of some of these experiments are shown in Table I. It is evident that although, on the acid side at least, the concentration of the negative ions is approximately that expected, the ratios of the positive ion concentrations are very far from correct and may even be in the wrong direction. This effect is more marked with Ca and Zn than with K. If the concentrations are corrected to activities by means of Lewis

[4] Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

TABLE I.

Distribution Experiment.

Comparison of Total Concentration Ratios with H^+ Activity Ratios.

Experiment.	Salt.	Concentration.	pH of gelatin.	H ratio: $\frac{\text{Filtrate}}{\text{Gelatin}}$.	Ion.	Ion ratio: $\frac{\text{Filtrate}}{\text{Gelatin}}$.	
						Observed.	Calculated from H ratio.
		M					
3/7	$CaBr_2$	0.005	2.83		Ca	1.84	8.4
	HBr	0.02		2.9	Br	0.446	0.344
3/14	KBr	0.004	3.75	3.63	K	3.55	3.63
	HBr	0.01			Br	0.26	0.275
	KBr	0.01	3.79	2.63	K	1.90	2.63
	HBr	0.01			Br	0.352	0.38
	KBr	0.02	3.81	1.66	K	1.34	1.66
	HBr	0.01			Br	0.475	0.60
3/19	$Ca(OH)_2$	0.00057	4.91		Ca	0.55	<1
	$CaBr_2$	0.004			Br	0.68	>1
	$Ca(OH)_2$	0.0027	5.84		Ca	0.32	<1
	$CaBr_2$	0.004			Br	0.91	>1
	$Ca(OH)_2$	0.0057	9.48		Ca	0.21	
	$CaBr_2$	0.004			Br	1.09	
	$CaBr_2$	0.004	4.79	0.76	Ca	0.75	0.58
	HBr	0			Br	0.59	1.32
	$CaBr_2$	0.004	4.31	1.90	Ca	2.57	3.6
	HBr	0.005			Br	0.41	0.52
	$CaBr_2$	0.004	2.42	2.3	Ca	2.35	5.3
	HBr	0.03			Br	0.465	0.43
	$CaBr_2$	0.004	1.95	1.6	Ca	2.15	2.56
	HBr	0.04			Br	0.50	0.62
	$CaBr_2$	0.004	1.70	1.4	Ca	1.93	1.96
	HBr	0.05			Br	0.56	0.71
3/27	$MgSO_4$	0.01	4.07	1.50	SO_4	0.51	0.45
	HBr	0.006			Br	0.69	0.66
					Mg	1.15	2.25

and Randall's ionic strength data,[5] the positive ion ratios become a little better but the negative ion ratios are worse. Evidently, either the Donnan equation does not hold under these conditions or a very appreciable amount of ions is combined with the protein. It will be noted that at pH 2.0 or lower, the calcium ratios become approximately correct. If the calcium combined with the same group as does the hydrogen, this would be the expected result since an excess of hydrogen ions would displace the calcium from its combination with the protein.

If these discrepancies are due to the combination of one of the ions of the salt with the protein then if the relative activities of the ions are determined by means of E.M.F. measurements, the correct values for the ratios should be obtained. Owing to the difficulty of obtaining satisfactory electrodes with alkaline earth or alkali metal electrodes, this experiment was performed with $ZnCl_2$.

Experimental.—40 gm. of dry powdered isoelectric gelatin were suspended in 500 cc. of solution containing the noted amounts of HCl and $ZnCl_2$, and stirred at 0° for 2 hours. Control experiments showed that this was sufficient time for equilibrium. The solution was then filtered and the filtrate analyzed for Zn and Cl. The volume of solution was noted and the concentrations of Zn and Cl in the gelatin were obtained by difference. The swollen gelatin was melted and the Zn, chloride, and hydrogen ion electrode potentials determined in the filtrate and melted gelatin at 25°, using a saturated KCl calomel electrode as a reference with a saturated KCl bridge and the usual potentiometric method. The Zn electrodes were prepared by amalgamating zinc rods. They gave satisfactory and reproducible results in aqueous solutions provided the solution was more acid than about pH 3.0. In the presence of gelatin the readings were constant without the addition of acid, but in acid solution the readings were low and increased slowly with time. This accounts for the discrepancy in the Zn potential in the acid solution. The chloride electrodes were prepared as described by MacInnes and Parker.[6] The electrode in the gelatin solution was negative to the electrode in the filtrate, in each case.

The activity of the ions in the solution, assuming no combination with the gelatin, was obtained from the total concentration by means of the table of Lewis and Randall,[5] connecting the ionic strength with

[5] Lewis, G. N., and Randall, M., Thermodynamics, New York and London, 1923, 382.

[6] MacInnes, D. A., and Parker, K., *J. Am. Chem. Soc.*, 1915, xxxvii, 1445.

the activity coefficient of the individual ions. It was assumed that the gelatin had no effect on the ionic strength but that the complex gelatin-hydrogen ion affected it in the same way as uncombined hydrogen ion. That is, the total HCl concentration was used to calculate the ionic strength and not simply the Cl concentration. It might be expected *a priori* that the protein would greatly affect the ionic strength of the solution, especially if, as seems probable, it is a polyvalent ion. It has been found, however, that the data are much more consistent if the assumptions mentioned above were used, *i.e.* that the pure protein has no effect and that the added HCl or other electrolyte affects the ionic strength in the same way as it would in the absence of gelatin. This amounts to assuming that the complex protein-hydrogen ion is monovalent.

The results of the experiment are given in Table II. The various electrode potentials agree within 1 mv., except in the most acid solution. In this case, as was stated above, the Zn electrode potential in the presence of gelatin is not constant. The ratios of the total concentrations of chloride ion are also quite close to the ion activity ratios, but the ratios of the total hydrogen ion and the total Zn ion concentrations are much too small; *i.e.*, the activities of the zinc and hydrogen ions in the gelatin are much smaller than would be expected from their total concentrations. The experiment shows directly, therefore, that the gelatin decreases the activity of the hydrogen and Zn ions but has little effect on that of the chloride ion. In the strongly acid solution there is no effect on the zinc ion when the correction for the ionic strength of the solution is made. These two facts render it very unlikely that the effect of the gelatin is upon the activity coefficient of the ions, since it would be expected that this would be apparent in all cases, but show that it is rather upon the concentration of the ions. That is, the hydrogen ion and the zinc ion combine with the gelatin to form complex ions which do not influence the electrodes. This has been well established in the case of hydrogen ions, and the present experiment shows that zinc behaves in an analogous way but to a smaller extent. The dissociation of the complex gelatin-Zn ion is evidently much greater than that of the gelatin-hydrogen ion.

The agreement between the various electrode potentials shows that the system obeys the Donnan equilibrium quite closely, and that the discrepancies obtained when total concentrations are compared are due to the formation of compounds between the gelatin and the ions.

This furnishes a method for the determination of the formation of such complexes without the use of E.M.F. measurements. The combination of any ion with a protein can be easily determined even though

TABLE II.

Comparison of Activity Ratios from Electrode Potential Measurements with Total Concentration Ratios. 40 Gm. of Gelatin. Total Volume 1,000 Cc. Total $ZnCl_2$ Concentration 0.01 M.

Experiment	1	2	3
Total HCl concentration	0.0105	0.0200	0.04
pH of gelatin	4.05	3.51	2.18
H electrode E.M.F., *mv*	15.5	20.0	14.5
Ag-AgCl electrode E.M.F., *mv*	15.2	19.0	15.0
Zn electrode E.M.F., *mv*	14.8	18.0	10.0
Ratio: Activity H in filtrate / Activity H in gelatin	1.83	2.19	1.76
Ratio: Total conc. H in filtrate (as HCl) / Total conc. H in gelatin (as HCl)	0.017	0.018	0.166
Ratio: Activity Cl in filtrate / Activity Cl in gelatin — calc. from H ratios	0.546	0.457	0.568
" " " observed E.M.F.	0.552	0.48	0.555
" " " analysis	0.508	0.42	0.48
Ratio: Total conc. Cl in filtrate / Total conc. Cl in gelatin	0.488	0.41	0.46
Ratio: Activity Zn in filtrate / Activity Zn in gelatin — calc. from H ratios	3.35	4.8	3.1
" " " observed E.M.F.	3.18	4.08	2.2
" " " analysis	2.11	3.23	3.0
Ratio: Total Zn conc. in filtrate / Total Zn conc. in gelatin	1.85	2.68	2.44

it is not possible to have an electrode for that ion. It is only necessary to determine the distribution in a system such as the above and also the activity ratio for some ion, such as hydrogen or chloride, which can be conveniently measured by E.M.F. measurements. Since the activity ratios of the other ions must be related to that of the chloride or hydrogen as expressed by Donnan's equation, the activity of the ion in the protein solution can be calculated from that of the outside solution. Lewis and Randall's tables of activity coefficients at

various ionic strengths furnish the data for determining this when the total concentration and ionic strength are known. The activity of the ion in the protein solution divided by its activity coefficient for a solution of the proper ionic strength gives the total concentration of the ion,[7] and this value subtracted from the total concentration as determined by analysis gives the amount combined with the protein.

The results of several experiments carried out in this way with $ZnCl_2$ are shown in Table III. They show that in 15 per cent gelatin at pH 4.7, over 50 per cent of 0.01 M Zn is combined with the gelatin and about 10 per cent of the chloride. It will be noted that in the first experiment the amount of Zn actually removed by the gelatin from the solution is just equivalent (1/2) to the chloride removed, since the outside solution must remain electrically neutral. If the particles were filtered, dried, and analyzed, it would be found, therefore, that they contained equivalent amounts of Zn and Cl and hence it might be concluded that the protein combined with both ions of the salt. This is, however, incorrect. Practically none of the chloride is combined and the remainder is simply held by electrostatic attraction. This illustrates the practical impossibility of obtaining reliable results as to compound formation by the analysis of protein precipitates. As the solution is made progressively more acid, less and less Zn is combined until at pH 2.0 there is practically none combined. The amount of chloride combined on the other hand increases about in proportion to the concentration, so that the per cent combined remains constant. The result bears out the idea, expressed before, that the Zn combines with the same group as does the hydrogen.

[7] This calculation necessarily involves a relation between the electrode potential of an ion and its concentration in the presence of other electrolytes. This is at present a very uncertain step, but it is possible to make the calculation by means of Lewis' ionic strength data. This method has been used throughout the present paper, although it is realized that such calculations are difficult for simple physical interpretation. The physical significance of the value obtained by dividing the activity of the Zn ion by its activity coefficient (as given by Lewis for a solution of that particular ionic strength) may be best expressed as the concentration of $ZnCl_2$ which would give the observed electrode potential in a solution of the same ionic strength. Since the amount of zinc found by analysis is in excess of this it seems logical to suppose that the excess of zinc is combined with the protein.

Determination of the Combination of Gelatin with Zn Chloride by Direct E.M.F. *Measurements.*

In order to confirm the results obtained by this method a series of experiments was made to determine the effect of gelatin on the potential of a zinc or chloride electrode in zinc chloride solution.[8] The

TABLE III.

Calculation of Zn and Cl Combined with Gelatin from Cl Activity Ratios. Total $ZnCl_2$ Concentration 0.01 M.

Experiment	1	2	3	4
pH of gelatin	4.7	4.05	3.51	2.18
Gm. of dry weight of gelatin per 100 cc. of swollen gelatin	19	14	11	9
Total concentration Zn in filtrate, M	0.0082	0.0115	0.013	0.014
" " Cl " " M	0.0178	0.0236	0.027	0.040
" " H " " M		0.0006	0.001	0.012
μ (ionic strength) of filtrate	0.025	0.035	0.039	0.053
γ Zn in filtrate	0.50	0.48	0.47	0.43
α Zn^{++} in filtrate	0.0042	0.0055	0.0060	0.0060
Ratio: $\frac{\alpha \text{ Cl in filtrate}}{\alpha \text{ Cl in gelatin}}$ (from Cl P.D.)	0.813	0.552	0.48	0.555
α Zn in gelatin (calculated)	0.0028	0.0018	0.0014	0.0018
Total H^+ in gelatin		0.036	0.055	0.075
" Zn " " observed	0.0126	0.0062	0.0048	0.0056
" Cl " " "	0.0253	0.0483	0.065	0.086
μ in gelatin	0.038	0.054	0.070	0.091
γ Zn in gelatin	0.47	0.42	0.39	0.35
C_{Zn} " " (calculated)	0.0059	0.0043	0.0036	0.0052
$C_{Zn\text{-gelatin}}$	0.0067	0.0019	0.0012	0.0004
Per cent Zn as Zn-gelatin	54	31	26	7
γ Cl in filtrate	0.88	0.86	0.86	0.84
α Cl " "	0.0156	0.020	0.023	0.0333
α Cl " gelatin (calculated)	0.0192	0.037	0.0466	0.060
γ Cl " "	0.86	0.83	0.82	0.80
C_{Cl^-} " "	0.0224	0.044	0.057	0.075
$C_{gelatin\text{-}Cl}$	0.003	0.0043	0.008	0.011
Per cent Cl as gelatin-Cl	11	9	12	12.5

zinc and Ag-AgCl electrodes were first standardized against a series of concentrations of zinc chloride made up in 0.001 M HCl, and the

[8] Measurements of Zn potentials in $ZnSO_4$-gelatin solutions have been made by Mutscheller (Mutscheller, A., *J. Am. Chem. Soc.*, 1920, xlii, 2142) who found that some Zn^+ but more SO_4^- was combined with the gelatin.

standard electrode potentials were calculated by using Lewis and Randall's figures for the ionic strength μ, and the activity coefficient γ. The values for the E.M.F. of the cell

$$Zn \mid M\ ZnCl_2 \mid \text{saturated KCl} \mid HgCl \mid Hg$$

and for the cell

$$Ag \mid AgCl \mid M\ ZnCl_2 \mid \text{saturated KCl} \mid HgCl \mid Hg$$

are shown in Table IV. All measurements were made at 25°. The experiment shows that the values obtained for the standard molar Zn

TABLE IV.

Zn-ZnCl₂ E.M.F. *Measurements.*

Concentration $ZnCl_2$.	Zn		$\gamma m = \alpha Zn$	pZn	E° observed.	$E^\circ = E$ obs. $-$ (0.0295 pZn)
	μ	γ				
M						
0.05	0.15	(0.32)	0.0162	1.79	1.0548	(1.002)
0.02	0.06	0.41	0.0082	2.086	1.063	1.0025
0.01	0.03	0.50	0.0050	2.301	1.070	1.002
0.005	0.015	0.57	0.00285	2.545	1.077	1.002
0.002	0.006	0.64	0.00128	2.893	1.087	1.0016
						Average.....1.0020
						Sat. KCl$-H_2$.0.243
						Zn, Zn^{++}; E°=0.7590

Ag-AgCl-ZnCl₂ E.M.F. *Measurements.*

Concentration Cl.	Cl		$\gamma m = \alpha Cl$	pCl	E observed.	$E^\circ = E$ obs. $+$ (0.059 pCl)
	μ	γ				
M						
0.041	0.060	0.83	0.0341	1.467	-0.070	0.0165
0.021	0.030	0.87	0.0183	1.737	-0.0850	0.0170
0.011	0.0155	0.90	0.0099	2.004	-0.1002	0.0176
0.005	0.0065	0.94	0.0047	2.328	-0.1190	0.017
						Average.....0.017
						Sat. KCl $-$ H_2.0.243
						Ag, AgCl, Cl^-; E° = -0.226

or Ag electrode against the saturated KCl calomel electrode are constant and independent of the salt concentration. The saturated calomel electrode used gave a calculated E.M.F. of $E = -0.243$ against

the normal hydrogen electrode, so that the value obtained for the standard Zn, Zn^{++} is 0.759 volt, and for the standard Ag, AgCl, Cl^- is − 0.226, which are within 1 mv. of the values given by Lewis and Randall.[9] This shows that the electrode measurements are reliable, and also that the data for the activity coefficients given by Lewis and Randall apply to $ZnCl_2$ solutions with considerable accuracy.

The experiments were then repeated with the addition of increasing concentrations of isoelectric gelatin. The results are given in Table V. The amount of Zn combined increases steadily and in the presence of 15 per cent gelatin amounts to 64 per cent. This agrees quite well

TABLE V.

Effect of Increasing Concentration of Gelatin on Zn and Chloride Activity of 0.01 M $ZnCl_2$.

Concentration of gelatin.	Zinc ($\gamma = 0.50$).					Chloride ($\gamma = 0.87$).				
	E.M.F. Zn	aZn	C_{Zn}	C_{Zn} combined with gelatin.	Per cent Zn combined.	E.M.F.	aCl	C_{Cl}	C_{Cl} combined.	Per cent Cl combined.
per cent								M		
0	1.070	0.0050	0.010	—	—	0.084	0.0183	0.021	—	—
1	1.072	0.00426	0.0085	0.0015	15	0.084	0.0183	0.021	—	—
2	1.073	0.00398	0.0078	0.0022	22	0.082	0.0183	0.021	—	—
5	1.076	0.00311	0.0062	0.0038	38	0.084	0.0183	0.021	—	—
10	1.081	0.00211	0.0042	0.0058	58	0.0852	0.0180	0.0201	0.0003	1
15	1.083	0.0018	0.0036	0.0064	64	0.0860	0.0172	0.0198	0.0012	6

with the results obtained from the distribution experiment (Table III). The amount of chloride combined is below the accuracy of the experiment until high concentrations of gelatin are reached, and then amounts to about 6 per cent.

The experiment was repeated using 10 per cent gelatin and different concentrations of $ZnCl_2$. The results are shown in Table VI. As would be expected and as is the case with acids, the amount combined increases with increasing Zn concentration but the per cent combined decreases.

The preceding experiments show that $ZnCl_2$ combines with gelatin in the same way as hydrogen chloride, but to a less extent. There

[9] Lewis and Randall, [5] pp. 407, 420.

seems no reason to suppose that this is other than ordinary complex ion formation; but, as in the case of hydrogen, experimental proof of the stoichiometric nature of the reaction is difficult to obtain. It can be shown, however, that the reaction is rapidly and completely reversible, which is the result expected if the compound is an ordinary chemical one and is the opposite of the result obtained in cases such as that of charcoal where there is surface adsorption. Table VII

TABLE VI.

Combination of Zn with Gelatin from Measurements with Zn Electrodes. 10 Per Cent Gelatin.

Concentration $ZnCl_2$.	E.M.F.	a_{Zn}	C_{Zn}	C_{Zn} combined with gelatin.	Per cent Zn combined.
M					
0.05	1.0595	0.011	0.0345	0.0155	31
0.02	1.0718	0.0043	0.0105	0.010	50
0.01	1.0812	0.0021	0.0042	0.0058	58
0.005	1.097	0.00095	0.00167	0.00383	76

TABLE VII.

Reversibility of Zn-Gelatin Combination.

	A	B
Method of preparing solution	50 cc. $\frac{M}{50}$ $ZnCl_2$ + 50 cc. 10 per cent isoelectric gelatin, 1 hr., 25°.	1 cc. $\frac{M}{1}$ $ZnCl_2$ + 25 cc. 20 per cent gelatin, 1 hr., 25° and then diluted to 100 cc.
E.M.F., Zn electrode	1.0760	1.0760
pH	4.70	4.70

shows that the activity of Zn^{++} and of H^+ are the same in the presence of the same concentration of gelatin whether the Zn and gelatin were mixed in concentrated solution and then diluted or whether they were diluted first.

Effect of the Combination with the Salt on the Properties of the Protein Solution.

If the gelatin combines more with one ion of the salt than with the other, as is indicated by the preceding experiments, and if the resulting compound is itself an ion, then the addition of $ZnCl_2$ to isoelectric

gelatin must give rise to ionized gelatin and hence to a Donnan equilibrium just as does the addition of acid, except to a much smaller extent. The osmotic pressure, swelling, and viscosity of gelatin should all be affected. That this is actually the case may be seen from Tables VIII, IX, and X. The effect is small, and in dilute gelatin solution

TABLE VIII.

Effect of $ZnCl_2$ on Viscosity of Gelatin Solutions. 5 Per Cent Isoelectric Gelatin.

Conc. $ZnCl_2$, M.	0.5	0.10	0.01	0
pH	3.95	4.33	4.55	4.8
Relative viscosity compared to corresponding aqueous solution of $ZnCl_2$ after 24 hrs. 37°	6.31	7.86	7.84	7.15

TABLE IX.

Effect of $ZnCl_2$ on Swelling of Particles of Isoelectric Gelatin. 8 Gm. of Dry Isoelectric Gelatin Added to 100 Cc. of $ZnCl_2$ and Kept at 2°C. for 18 Hours. Total Volume 106 Cc.

Conc. $ZnCl_2$, M.	0.5	0.10	0.01	0
Volume of filtrate (average of 3 experiments)	49	53	55	59
" " gelatin	57	53	51	47

TABLE X.

Effect of $ZnCl_2$ on Osmotic Pressure of Gelatin. 25 Cc. of 10 Per Cent Isoelectric Gelatin in Collodion Sac Suspended in 60 Cc. of Various Concentrations of $ZnCl_2$ pH 4.7 at 37°. Readings after 24 Hours.

Conc. $ZnCl_2$, M.	0	0.02	0.20	0.50
Osmotic pressure in mm. Hg (average of 6 experiments)	37.7±1.0	56±1.8	53.0±0.8	48.2±0.6
Chloride ratio (from E.M.F.): $\frac{\alpha \text{ Cl inside}}{\alpha \text{ Cl outside}}$		1.20		
α Zn^{++} ratio, calculated from Cl ratio		0.70		
α Zn^{++} " " " total Zn concentration		1.20		

would be negligible compared to the effect of acid. The fact that the Zn is more concentrated inside the membrane than outside shows, however, that the result is not due to the H^+, since if the Zn were merely inert the concentration of Zn outside would necessarily be greater than inside in order to agree with the H^+ and Cl^- ratios.

The osmotic pressure experiment is the most direct, since both the swelling and viscosity are influenced by other factors than the ion concentration. The swelling depends also on the "cohesion" of the gelatin particles; if the force which tends to hold the particles to the original size should decrease this swelling will evidently increase although the osmotic pressure may be no greater. This presumably accounts for the fact that the swelling maximum does not agree with the maximum of osmotic pressure.

The viscosity is a function of time and the selection of the proper value is difficult. The figures given were obtained after 24 hours at 34° and were constant for about 10 hours. Before this time, the control and most concentrated solutions decreased in viscosity while the intermediate concentrations increased.

It will be noted in Table VIII that the pH of the solutions becomes lower as Zn is added, although both solutions were originally pH 4.7. Diluting either the gelatin or $ZnCl_2$ with water does not cause a change in pH. The effect is therefore due to a reaction between the $ZnCl_2$ and gelatin. If, as was suggested, the Zn replaces the H from combination this increase in acidity is due to the liberated H^+ just as adding acid was found to increase the Zn^{++} concentration.

SUMMARY.

1. It has been found that the ratios of the total concentrations of Ca, Mg, K, Zn, inside and outside of gelatin particles do not agree with the ratios calculated according to Donnan's theory from the hydrogen ion activity ratios.

2. E.M.F. measurements of Zn and Cl electrode potentials in such a system show, however, that the ion activity ratios are correct, so that the discrepancy must be due to a decrease in the ion concentration by the formation of complex ions with the protein.

3. This has been confirmed in the case of Zn by Zn potential measurements in $ZnCl_2$ solutions containing gelatin. It has been found that in 10 per cent gelatin containing 0.01 M $ZnCl_2$ about 60 per cent of the Zn^{++} is combined with the gelatin.

4. If the activity ratios are correctly expressed by Donnan's equation, then the amount of any ion combined with a protein can be determined without E.M.F. measurements by determining its distribution

in a proper system. If the activity ratio of the hydrogen ion and the activity of the other ion in the aqueous solution are known, then the activity and hence the concentration of the ion in the protein solution can be calculated. The difference between this and the total molar concentration of the ion in the protein represents the amount combined with the protein.

5. It has been shown that in the case of Zn the values obtained in this way agree quite closely with those determined by direct E.M.F. measurements.

6. The combination with Zn is rapidly and completely reversible and hence is probably not a surface effect.

7. Since the protein combines more with Zn than with Cl, the addition of $ZnCl_2$ to isoelectric gelatin should give rise to an unequal ion distribution and hence to an increase in swelling, osmotic pressure, and viscosity. This has been found to be the case.

OBSERVATIONS ON THE INJURIOUS EFFECTS OF HIGH TEMPERATURES ON FROG SKIN.

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(Accepted for publication, June 22, 1924.)

It has been shown by Osterhout (1) that the electrical conductance of living tissues may be used as a general criterion of their vitality, and may in particular be employed in quantitative studies of injuries inflicted upon them. It has been found by this investigator that such injuries may give rise to one of three results, namely (*a*) progressive change leading to death, (*b*) partial recovery, or (*c*) complete recovery. These studies of Osterhout suggest the possibility of using in a similar way some other electrical property of tissues in attempting to define such indefinite terms as vitality, injury, and death. Thus, for example, it has been known since the early work of Du Bois-Reymond (2) that there is a difference of electrical potential between the inner and outer surfaces of the skin of the frog; and this potential difference has been the subject of much investigation since that time.[1] The question naturally arises how far the results of Osterhout might be paralleled by those obtained by studies on it; and the observations here reported were made with the object of determining this point.

The method employed was as follows: The skin, having been removed from various parts of the pithed animal with a minimum of trauma, was cut into squares of sufficient size to fit without undue stretching over the flanged end of a short glass cylinder of about the caliber of an ordinary test-tube, and was held in place there by a rubber band. The tube was then filled with Ringer's solution, and its skin-covered end immersed in a dish of the same solution. Non-polarizable $Zn\text{-}ZnSO_4$ electrodes served as leads from each of these two solutions, and the

[1] More extensive references to the literature on this and certain other points will be given when the complete data are published in a later paper.

difference of potential between them was measured by means of an accurate potentiometer. Precautions were taken to see that the two electrodes themselves were at the same potential at the beginning and during the course of the experiments.

Measurements made in the manner described have confirmed the observations of others that when Ringer's solution is used on both sides of the skin, the external surface is always electronegative to the internal, and none of the procedures used in these experiments sufficed to reverse this polarity. The skin of the large bullfrog (*Rana catesbiana*) obtained at Woods Hole showed considerably higher P.D. than those recorded by most previous observers who have usually obtained values from 30 to 90 mv. The present observations on freshly captured bullfrogs gave a range of from 55 to 141 mv., though values of the usual magnitude were obtained with ordinary laboratory frogs in Philadelphia.

That there is some correlation between the vitality of the skin and its observed P.D. is indicated in a general way by the fact that sickly frogs and those held captive a long time usually give much lower values than do healthy ones recently captured. Similarly, it is easy to show that various noxious agents which are generally admitted to damage the skin (*e.g.* drying, formalin, alcohol, strongly hypertonic salt solutions, etc.) cause a progressive drop in the P.D., leading, finally, to a zero potential. While it is thus possible to use for purposes of study a variety of injurious agents, high temperatures alone were employed in these experiments because of the ease with which they can be controlled and measured.

The nature of the effects produced by continuous exposures to high temperatures was first studied, and the results from one typical experiment are reproduced in Fig. 1. For purposes of more ready comparison the readings have been changed from millivolts to percentages of their value at the beginning of the experiment. This device was utilized because it usually is not possible to obtain, even from the same animal, a number of pieces of skin the P.D. of which are exactly the same. It will be noted that the control curve for room temperature follows a somewhat erratic course (see also Fig. 2). This is typical; and the most common result is a more or less regular fall in the P.D. toward zero. Usually, with the skin on the tubes in

Ringer's solution, this point has not been reached in 24 hours; and the skin may be kept for this length of time on the dead animal without entirely losing its P.D. if precautions are taken to keep it moist.

It will be observed that with the exception of the one representing the lowest temperature, where there was a short characteristic preliminary rise, the curves all show a progressive fall, rapid at first, but becoming slower and slower as the zero point is approached.

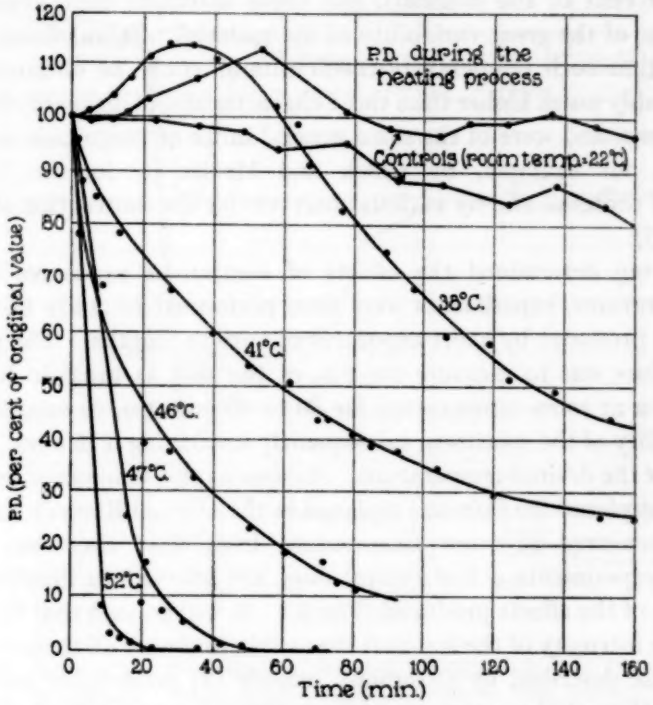

FIG. 1. Course of the P.D. of frog skin during continuous exposure to injurious temperatures. Each curve represents a single experiment.

In certain cases a good straight line relation may be obtained by plotting the logarithm of the P.D. (measured in per cent of its original observed value in millivolts) against time; but the author does not wish to draw the conclusion that a monomolecular reaction is necessarily involved, since many purely physical processes are known to follow the same law. Brooks (3) and others have warned against the mistaken resemblance of the time curves of a number of destruc-

tive biological processes to that for monomolecular reactions, pointing out that the apparent logarithmic straight lines are in reality S-shaped curves. Some of the logarithmic curves obtained from the present experiments show this latter relation. A number of attempts have been made to determine in an exact manner a temperature coefficient for the injurious effects of heat by comparing the times required for different temperatures to depress the P.D. to a given level (*e.g.* 50 per cent of the original); but these attempts were abandoned because of the great variability of the material. It was found, however, that such temperature coefficients as could be obtained were invariably much higher than those characteristic of ordinary chemical reactions, and were of the same general order of magnitude as those found, for example, by Chick and Martin (4) for the coagulation of proteins, and by various observers for the destruction of living cells (5).

Having determined the effects of continuous exposures to high temperatures, experiments were then performed to study the after-effects produced by short exposures of various lengths. The general procedure was to measure the P.D. of the skin as usual in Ringer's solution at room temperature for 30 to 40 minutes, to establish the normality of the specimen, subsequently immersing it in a hot Ringer bath at the desired temperature. As soon as this temporary exposure was completed the skin was replaced in the original Ringer's solution, and measured at room temperature from that time on. From these experiments a few typical ones are selected to illustrate the nature of the effects produced (Fig. 2). It will be seen that by varying the intensity of the injury it is possible to obtain all three types of response described by Osterhout, namely (*a*) progressive decline to death, (*b*) partial recovery, and (*c*) complete recovery. It is impossible in a figure of this size to continue the curves beyond a point representing 200 minutes from the beginning of the experiment, but the author has followed such experiments for many hours without obtaining further changes of significance. In addition to the method thus described another has been used in some experiments, namely varying the time of the exposure with the temperature constant, instead of using the temperature as the variable. The results, while somewhat less regular than those already described, nevertheless have been of the same nature.

The results obtained, therefore, parallel in a general way those of Osterhout. The P.D. of frog skin may to a certain extent be used as a criterion of injury, and from measurements of its magnitude at intervals curves of recovery and death may be constructed. It must be admitted, however, that for purposes of quantitative study involving the exact nature of the processes of death and recovery the method is

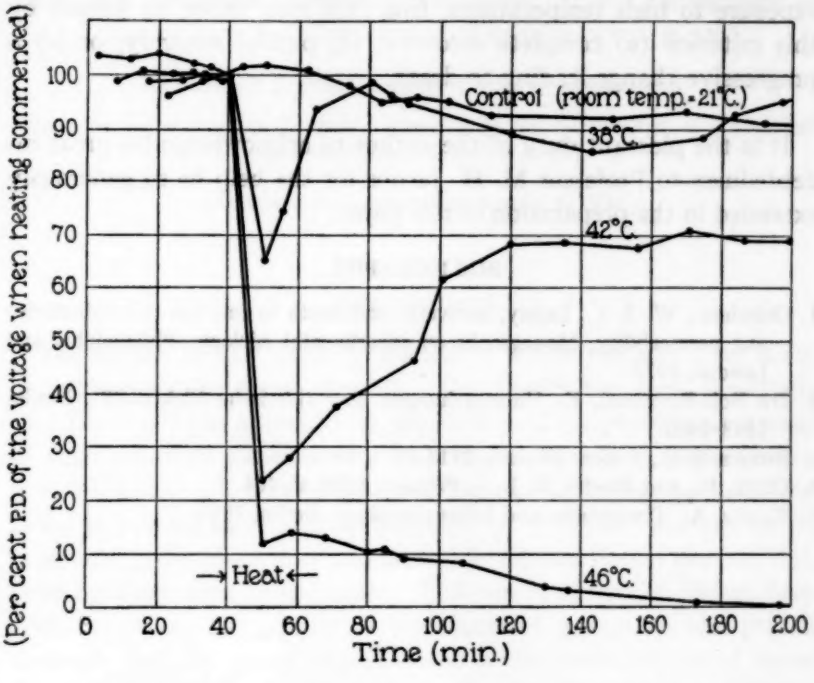

FIG. 2. Showing the after-effects of temporary exposure to injurious temperatures for 10 minutes. (Heat applied from the 40th to 50th minutes.) Each curve represents a single experiment.

inferior, at least in the hands of the author, to the electrical conductance method of Osterhout. By reason of its more limited applicability, and because of the greater complexity and variability of the material, the method herein described yields results which are not easily reproducible; though it may perhaps lay some claim to value as an independent and supplementary means of testing the conclusions of previous workers.

SUMMARY.

1. The P.D. between the two surfaces of the skin of the frog, when measured under standardized conditions, may be used as a means of studying the phenomena of injury, recovery, and death. Injurious conditions decrease the P.D.

2. According to the severity of the injury produced by temporary exposure to high temperatures, frog skin may show, as judged by this criterion (*a*) complete recovery, (*b*) partial recovery, or (*c*) a progressive change leading to death.

It is the pleasant duty of the author to acknowledge his great indebtedness to Professor M. H. Jacobs for the help he so generously extended in the preparation of this paper.

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STUDIES IN THE PHYSICAL CHEMISTRY OF THE PROTEINS.

III. The Relation between the Amino Acid Composition of Casein and Its Capacity to Combine with Base.*

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(Accepted for publication, August 4, 1924.)

I.

INTRODUCTION.

The nature of the forces by means of which proteins bind alkali have given rise to many theories. One theory has been the theory that the alkali was adsorbed on the surface of the proteins as a result of their colloidal dimensions. This theory has been made far less probable as a result of investigations demonstrating that proteins combined with acids and bases in equivalent proportions,[1] and has for the most part given place to a stoichiometric explanation of the combinations between proteins and bases. Differences of opinion obtain, however, regarding the nature of the chemical groups in the protein molecule that are specifically involved in the neutralization of bases. Two views have generally been held. The one, that the groups of such dicarboxylic amino acids as glutamic acid, which were not held in polypeptide linkage, were involved in the neutralization of bases and the formation of protein compounds. The other view, advanced by

* A report of this investigation was read before the XI. International Physiological Congress, held in Edinburgh, July 23–27, 1923 (1).

[1] The accumulated evidence has become too well known, and too extensive to warrant detailed references, in this place, to more than a few investigations in which electromotive force measurements were made: D'Agostino, E. and Quagliariello, G. (2), Pauli, W. (3), Robertson, T. B. (4), Sörensen, S. P. L. (5), Henderson, L. J. and Cohn, E. J. (6), and Loeb, J. (7).

Robertson (4) among others, was to the effect that there were not nearly enough terminal carboxyl groups in such a protein as casein to account for its base-combining capacity, and that the opening of internal – COHN – groups must, therefore, be involved in the neutralization of bases.[2]

Many proteins contain more dicarboxylic acids than earlier investigators succeeded in isolating. By means of his butyl alcohol method of extracting amino acids Dakin (9, 10) has not only increased the yields of glutamic and aspartic acids from the hydrolysates of a number of proteins, but has also discovered a new dicarboxylic amino acid, β-hydroxyglutamic. The content of dicarboxylic acids revealed by earlier investigators was in many cases not greater than the ammonia content, and the conclusion was, therefore, drawn that the groups of dibasic acids that were not held in polypeptide linkage were for the most part bound as amides (11). This view was difficult to reconcile with the acidic nature of many proteins. The newer analyses (9) have increased the yields of dicarboxylic amino acids isolated from casein from 16.9 to 36.4 per cent. In short, the yield of these divalent acids in casein has been more than doubled by these recent analyses which indicate that the dicarboxylic acids in casein, and in many other proteins, are far in excess of the ammonia (12, 13). As a result, casein must possess a number of terminal carboxyl groups.

This investigation was undertaken in order to determine whether the number of terminal carboyxl groups that could exist in conformity with this conception of the structure of casein was sufficient to account for its base-combining capacity. Robertson (14, 4[3]) had previously determined the base-combining capacity of casein. Our increased knowledge of the composition and of the molecular weight of casein rendered it desirable at this time to redetermine this quantity.

In a recent study of the relation between the solubility of casein and its capacity to combine with base in the neighborhood of its isoelectric point, we showed that each mol of base added to casein

[2] At the same time that these investigations were nearing completion a new study of Robertson (8) appeared in which he "inferred that the predominant method of union of proteins with acids and bases is not through the agency of free amino or carboxyl groups."

[3] Robertson (4), p. 198.

carried an equivalent amount of the protein into solution. Specifically each mol of sodium hydroxide combined in our experiments with between 2,100 and 2,150 gm. of casein. This is just twice the combining weight of casein estimated by a very different method by Robertson (4), and by Van Slyke and Bosworth (15, 16), and one-half the molecular weight postulated by them for casein from its sulfur and phosphorus content.

The amino acids that casein yields upon hydrolysis suggest that the molecular weight of casein must be greater than 4,300. From a consideration of the many analyses that have been made, of the different amino acids in casein, often by different investigators, we came to the conclusion (13) that the molecular weight of this protein could not be less than 12,800, and might be some multiple of this figure. 12,800 was suggested because it was three times the molecular weight previously postulated by Robertson, and by Van Slyke and Bosworth, from the sulfur and phosphorus content of casein; six times the equivalent combining weight of casein for base determined by us; and was at the same time the smallest molecular weight that could be ascribed to casein provided each molecule of the protein contains one molecule of tryptophane. The sulfide sulfur and the cystine content of casein suggest that the true molecular weight of casein may be seven or eight times this value. A consideration of these data will be reserved for a study (Paper V of this series) of the molecular weights of certain proteins.

II.

The Amino Acids in Casein.

We shall consider the molecular weight of casein as 12,800 in the following calculations. It should be pointed out, however, that no error whatsoever is introduced into the calculation of the base bound by a protein by this, or any other, choice of a unit. Thus, in Table I, the base bound by 1 gm. of casein has been calculated from the percentage composition, without any assumption regarding the molecular weight. The stoichiometric relations involved are more apparent, however, if we refer base-combining capacity to equivalent, or molecular, rather than to absolute weight of the protein.

On the basis of a molecular weight of 12,800, 1 molecule of casein must contain at least 19 molecules of glutamic acid, 4 molecules of

aspartic acid, and 8 molecules of β-hydroxyglutamic acid. The method of calculation and of tabulation that has been followed has been explained in our previous communication (13)[4] from which Table I is derived. Assuming that the dicarboxylic acids were in polypeptide linkage and that the ammonia yielded by casein represented amide groups, the 12 molecules of ammonia that casein contains must be subtracted from the number of dicarboxylic acids in order to give the number of available terminal carboxyl groups. This has been

TABLE I.

Calculation of Free Acid Groups in Casein.

	Dicarboxylic amino acids in casein.				Amide-bound dicarboxylic acids.	Free dicarboxylic acids.	Phosphorus in casein.	Total free acid groups.
	Glutamic acid.	Aspartic acid.	β-Hydroxyglutamic acid.	Total.	Ammonia.			
	(*a*)	(*b*)	(*c*)	(*a*+*b*+*c*)	(*d*)	(*a*+*b*+*c*−*d*)	(*e*)	(*a*+*b*+*c*−*d*+3*e*)
Found in casein, *per cent*.....	21.77	4.1	10.5	36.37	1.61		0.71	
Mols in 1 gm. of casein × 10^{-5}..	148.0	30.8	64.4	243.2	94.5	148.7	22.9	217.4
Weight of casein containing 1 mol, *gm*.....	675	3,246	1,550		1,060		4,372	
No. of assumed mols.....	19	4	8	31	12	19	3	28
Calculated molecular weight....	12,825	12,984	12,400		12,720		13,116	

done in Table I. From this source, therefore, one might expect to find nineteen strong terminal carboxyl groups in the casein molecule.

The number of terminal groups may be larger than this estimate. It can scarcely, however, be smaller. It is probable that the phosphorus in casein represents phosphate phosphorus, and that one or more of the acid groups of phosphoric acid may be free in the casein molecule. It is possible that further advances in the methods of extracting and separating the amino acids may increase even the high yields that

[4] Cohn and Hendry (13), p. 547.

Dakin has now obtained. It is conceivable that such amino acids as tyrosine and cystine may retain a certain capacity to combine base when held in polypeptide linkage. Finally certain monoamino monocarboxylic acids may be so oriented in the protein molecule as to yield terminal groups. On the other hand, certain of the dicarboxylic acids might exist in such firm combination with the terminal groups of diamino acids that they would not bind base at the alkalinities that we have reached. These considerations indicate that the maximal number of terminal base-binding groups in casein can be greater, but not very much greater, than nineteen. Even if all of the valences of phosphoric acid were either free, or, through combination with amino groups, had liberated carboxyl groups from polypeptide linkage, the number of groups only becomes twenty-eight. On the basis of a molecular weight of 12,800 we would therefore expect casein to possess between nineteen and twenty-eight free acid groups. Each gram of casein would, if these conditions obtained, combine between 148.7 and 217.4 $\times 10^{-5}$ mols of base.

III.

The Measurement of Base-Combining Capacity.

The base bound by protein in alkaline solution can be estimated with great accuracy from the decrease in the concentration of free base. If one knows the original concentration of the basic solution, and the amount of the protein it contains, the determination of the residual free base suffices for the calculation of the base bound by the protein. The free base in solution can be directly determined by electromotive force measurements of the hydrogen electrode against any known standard.

Our hydrogen electrode measurements are referred to the 0.1 N calomel electrode. In the course of the investigation our standard electrode has been checked against seven 0.1 normal and six saturated calomel electrodes. A saturated potassium chloride salt bridge has been used throughout these experiments and no correction has been applied for the liquid junction potential. Our results have been calculated by the equation

$$pH^+ = \frac{\text{E.M.F. observed} - \text{E.M.F. 0.1 N calomel}^5}{0.001983\ T} \quad (1)$$

From the hydrogen potential, or pH^+, the hydroxyl potential, pOH^-, can be calculated, since their sum is equal to pK_w, the negative logarithm of the dissociation constant of water.

$$pH^+ + pOH^- = pK_w \quad (2)$$

In any investigation involving alkaline solutions it is far more convenient to consider the hydroxyl than the hydrogen potential. For "although the pH^+ of bases changes greatly with the temperature, since the dissociation constant of water changes greatly, their pOH^- is nearly independent of the temperature" (20).[6] Our measurements of the hydroxyl potentials of sodium hydroxide solutions, made at different temperatures, confirm this contention.

The hydroxyl potential is a measure of the free hydroxyl ions in solution. It is usually defined by the equation

$$pOH^- = \log \frac{1}{(OH^-)} \quad (3)$$

and may be so defined, provided by (OH^-) we imply either the stoichiometric concentration of hydroxyl ions, or their activity (21). Whether we are actually measuring the hydroxyl ion concentration in strong sodium hydroxide solutions, or the activity of the hydroxyl ions is merely a matter of terminology. In practise we measure the

[5] The electromotive force of the 0.1 N calomel electrode is in doubt by 2 mv. Up to the present Sörensen's (17) value of 0.3376 volt for this half cell has always been used in this laboratory. In 1917 Lewis, Brighton, and Sebastian (18) measured the electromotive force of the normal calomel electrode. From their value Clark (19) has estimated the E.M.F. of the 0.1 N calomel electrode at 25°C. to be 0.3356 volt. Clark has suggested, however, that Sörensen's values "be used as provisional standards whenever there is no definite reason to require any other value."

Measurements that have been made in this laboratory of hydrochloric acid and sodium hydroxide solutions of concentrations between 0.001 N and 0.1 N have led us to adopt Lewis, Brighton, and Sebastian's value for the calomel electrode, at least tentatively, and to define the constants involved in our calculations in a slightly different way than has been customary.

[6] Michaelis (20), p. 21.

hydroxyl potential, and determine its functional relation to the stoichiometric concentration of sodium hydroxide.

Since the earliest measurements of the conductivity of solutions it has always been apparent that as the concentration of strong electrolytes increased their equivalent conductance decreased. The relation between their stoichiometric concentration and the apparent concentration of their ions has always been expressed by an equation of the type

$$\frac{(OH^-)}{(NaOH)} = \gamma \tag{4}$$

In terms of the theory of partial dissociation γ is called the degree of dissociation. In terms of the theory of complete dissociation it is called the activity coefficient. Without subscribing to any theory we can relate the hydroxyl potential of a solution of sodium hydroxide to its concentration in terms of γ by combining equations (3) and (4)

$$pOH^- = \log \frac{1}{(NaOH)\gamma} = pNaOH + p\gamma \tag{5}$$

The convenient symbols, pNaOH and $p\gamma$, are defined as negative logarithms of (NaOH) and γ, respectively.

In very dilute solutions γ is usually considered equal to 1, and the hydroxyl ion concentration is therefore directly proportional to the sodium hydroxide concentration. In more concentrated solutions, however, γ becomes progressively smaller. In order accurately to calculate sodium hydroxide concentrations from hydroxyl potential measurements the activity coefficient, γ, must be known.

Various methods of estimating γ for hydrochloric acid and sodium hydroxide have been attempted by different investigators. Values derived from conductivity measurements have led to higher values of γ than those derived from the measurements of freezing point lowering, or of electromotive force. Regardless of the method employed in estimating this quantity, however, its value increases with dilution; and the values for different electrolytes of the same valence type approximate each other in dilute solution. As a result it is relatively certain that γ values of sodium hydroxide and hydrochloric acid are essentially identical at low concentrations. Upon the coincidence in the values

TABLE II.

Calculation of the Hydrogen Potential from the Activity Coefficient and the Concentration of the Hydrogen Ion.

Molality. (m)	Activity coefficient. (Lewis and Randall). (γ)	$\log \frac{1}{m}$ (pm)	$\log \frac{1}{\gamma}$ ($p\gamma$)	pH^+ calculated. ($pm + p\gamma$)	pH^+ determined. Lewis, Brighton, and Sebastian's value for calomel.	pH^+ determined. Sörensen's value for the calomel electrode.
0.001	0.98	3.000	0.009	3.009	3.016	2.980
0.002	0.97	2.699	0.013	2.712		
0.005	0.95	2.301	0.022	2.323		
0.010	0.92	2.000	0.036	2.036	2.029	1.994
0.020	0.90	1.699	0.046	1.745	1.739	1.705
0.025		1.602	(0.048)	1.650	1.649	1.615
0.030		0.523	(0.050)	1.573		
0.040		1.398	(0.053)	1.451	1.463	1.428
0.050	0.88	1.301	0.056	1.357	1.363	1.329
0.100	0.84	1.000	0.076	1.076	1.064	1.030

Calculation of the Hydroxyl Potential from the Activity Coefficient and the Concentration of the Hydroxyl Ion and the Dissociation Constant of Water.

Molality. (m)	Activity coefficient. (γ)	$\log \frac{1}{m}$ (pm)	$\log \frac{1}{\gamma}$ ($p\gamma$)	pOH^- calculated. ($pm + p\gamma$)	pOH^- determined. Lewis, Brighton, and Sebastian's value for calomel and for K_w.	pOH^- determined. Sörensen's value for calomel and for K_w.	pOH^- determined. Lewis, Brighton, and Sebastian's value for calomel and Sörensen's for K_w.
0.001	0.98	3.000	0.009	3.009			
0.002	0.97	2.699	0.013	2.712			
0.005	0.95	2.301	0.022	2.323			
0.010	0.92	2.000	0.036	2.036	2.160	2.058	2.024
0.020	0.89	1.699	0.051	1.750			
0.025		1.602	(0.055)	1.657	1.795	1.693	1.659
0.030		1.523	(0.059)	1.582			
0.040		1.398	(0.065)	1.463			
0.050	0.85	1.301	0.071	1.372	1.497	1.395	1.362
0.100	0.81	1.000	0.091	1.091	1.227	1.125	1.091

of γ for sodium hydroxide and hydrochloric acid at concentrations from 0.001 N to 0.010 N we propose to base the choice of the values for the constants involved in these calculations.

Lewis and Randall (21) have recently estimated the activities of the hydrogen and hydroxyl ions from E.M.F. measurements.[7] By means of equation (5) we have calculated the hydrogen potentials of hydrochloric acid and the hydroxyl potential of sodium hydroxide solutions from their results (Table II). They indicate that at 0.01 N, and at lower concentrations, the hydrogen potential is equal to the hydroxyl potential.

In the course of the present investigation a large number of measurements has been made of the hydrogen potentials of hydrochloric acid, and the hydroxyl potentials of sodium hydroxide solutions in precisely the same way that we have measured the potentials of similar solutions containing proteins. The averages of these results are included in Table II, where the E.M.F. measurements have been calculated both by means of Sörensen's and of Lewis, Brighton, and Sebastian's values for the 0.1 N calomel electrode and for the dissociation constant of water.[8]

[7] Chow (22) and Knobel (23) have studied the activities of potassium hydroxide solutions. Ellis (24) has studied the activity of hydrochloric acid solutions, and Lewis and Randall (21) and MacInnes and Parker (25) have studied the activities of potassium chloride solutions. The activities of the hydrogen and hydroxyl ions can be estimated from these data, if the activity of the potassium and chlorine ions are considered identical, by subtracting the $p\gamma$ value of potassium chloride from twice the $p\gamma$ value of hydrochloric acid or potassium hydroxide. Combination of the results of these different investigators leads to slightly different values for the activity of the hydrogen and hydroxyl ions.

[8] If we adopt Sörensen's value for the 0.1 N calomel electrode in calculating these results we come to the impossible conclusion that the activity of the hydrogen ion is 1 at 0.01 N and greater than 1 at 0.001 N. If, however, we employ the value for this half cell, calculated by Clark from Lewis, Brighton, and Sebastian's study of the normal calomel electrode, we obtain values for the hydrogen potential in close agreement with those calculated from Lewis and Randall's coefficients for the activity of the hydrogen ion. It is for this reason that we have adopted, tentatively, the latter value.

The calculation of the hydroxyl ion concentrations from hydrogen electrode measurements is further complicated by uncertainty regarding the dissociation constant of water. Michaelis (20) has recalculated Sörensen's results for different temperatures, and gives 1.27×10^{-14} as the value for this constant at 25°C. This result is in close agreement with the value of Lorenz and Böhi (26). These investigators studied the same system as Lewis, Brighton, and

The determination of the base bound by proteins is in no way affected by the constant employed either for the calomel electrode or for the dissociation constant of water. For the determination of base-bound involves the measurement of the hydroxyl potentials both of sodium hydroxide solutions, and of sodium hydroxide solutions containing protein, and the constants are eliminated in the calculation. In practise, however, we have found it convenient to adopt the values for these constants that brought our data into conformity with the more extensive activity coefficients of Lewis and Randall, so that we might use them in subsequent calculations.

IV.

The Calculation of Base-Combining Capacity.

The greatest source of error in estimating the base-combining capacity of a protein inheres in uncertainty regarding the state of the base bound by the protein. We know that when we add a protein to an alkaline solution base is bound, and the hydroxyl potential increases. In the previous section we estimated the hydroxyl potentials of sodium hydroxide solutions. These indicated that the activity of the hydroyxl ion changes appreciably over the range of concentrations with which we are now concerned.

Sebastian, who obtained the same E.M.F. for the chain. The corrections employed by them for the activity of the ions and for the diffusion potentials appear more reliable. Lewis and Randall's recalculation yields the value 1.005×10^{-14} for K_w. Although this is now the most probable value for the dissociation constant of water, its use in the calculation of our data leads to different values (Table II, Column 6) for pOH^- in alkaline solution than those calculated from Lewis and Randall's coefficients for the activity of the hydroxyl ion. In all probability this depends not upon the value of K_w, but upon diffusion potentials, for which we have not corrected. If however, we employ the Sörensen-Michaelis values for K_w at different temperatures, our pOH^- values conform approximately to those calculated from the activity of the hydroxyl ion. Moreover, at lower concentrations than 0.01 N the pH^+ in hydrochloric acid solutions is then equal to the pOH^- in sodium hydroxide solutions. Without making any assumption whatever as to the correct value for K_w we have tentatively adopted the latter in conformity with the assumption that in very dilute solution the activity coefficient of the hydrogen ion, however measured, is equal to that of the hydroxyl ion.

The fact that in these experiments the increase in hydroxyl potential is produced by protein renders it uncertain, however, in what way to apply a correction for the activity of the hydoxyl ion. All of the sodium present in the original sodium hydroxide solution was still present in these systems. Had we added hydrochloric acid instead of casein and formed sodium chloride instead of a sodium compound of casein we should assume that the activity of the hydroxyl ion had not been appreciably affected since the total concentration of sodium had not changed (21).[9] Whether the sodium bound by casein is as free to affect the activity of the hydroxyl ion as that bound by hydrochloric acid can, perhaps, not be decided *a priori*.

That the base bound by amino acids has no effect upon the activity of the hydroxyl ion has, however, been tacitly assumed by Tague (27) and more recently by Harris (28). Tague believed that "the hydrogen electrode makes possible an exact differentiation between an amount of alkali or acid to be apportioned to the solvent and solute, respectively, at any pH value."[10] "Since at each pH value the concentration of the OH^- ion both in the blank and in the solution of the amino acid are the same and the volume of the original solution and the blank are equal, there would be no difference in ionization in the observed results for the blank and for the solution of amino acid."[11] As a "blank" Tague used a solution of sodium hydroxide that gave the same pH^+ as the more concentrated sodium hydroxide solution that contained the amino acid.

It seems necessary to point out the fallacy in this type of reasoning since Tague's method has also been applied to the study of protein solutions. In the first place the hydrogen electrode measures the activity of the hydroxyl ion, as has been indicated, and not its concentration. In the second place the assumption that the base bound by an amino acid has no effect upon the activity of the hydroxyl ion is untenable. Since this hypothesis demands that amino acids behave in a very different way than other electrolytes, we have tested it experimentally. We partially neutralized an 0.05 N solution of sodium hydroxide with 0.04 N hydrochloric, acetic, and amino acetic acid—

[9] Lewis and Randall (21), p. 368.
[10] Tague (27), p. 183.
[11] Tague (27), p. 182.

glycine—and determined the hydroxyl potentials (Table III). The concentration of sodium hydroxide was in each case 0.01 N. By subtracting the negative logarithm of the sodium hydroxide concentration, pNaOH, from the hydroxyl potential, pOH^-, the negative logarithm of the activity coefficient, $p\gamma$, was obtained

$$pOH^- - pNaOH = p\gamma \quad (6)$$

The hydroxyl potentials of two sodium hydroxide solutions are included in Table III for comparison. The one contained the same

TABLE III.

The Activity Coefficient of the Hydroxyl Ion in Sodium Hydroxide Solutions Containing Other Sodium Compounds.

	NaOH	NaOH	NaCl	CH_3COONa	$C_2H_4NO_2Na$	$Na_2C_2O_4$	Na_2SO_4	$C_6H_7NO_2Na$	$C_6H_4NO_4Na$
NaOH conc. N	0.01	0.05	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Salt conc. N	0.00	0.00	0.04	0.04	0.04	0.04	0.04	0.04	0.04
Na^+ conc. N	0.01	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
$\text{Log } \frac{1}{(NaOH)}$	2.000	1.301	2.000	2.000	2.000	2.000	2.000	2.000	2.000
$\text{Log } \frac{1}{(OH^-)}$	2.026	1.362	2.081	2.073	2.064	2.092	2.088	2.073	2.084
$\text{Log } \frac{1}{\gamma}$	0.026	0.061	0.081	0.073	0.064	0.092	0.088	0.073	0.084
γ	0.94	0.87	0.83	0.85	0.86	0.81	0.82	0.85	0.82
γ (Lewis and Randall)	0.92	0.85							

concentration of free hydroxide, the other of total sodium, as the solutions containing salts. It will be noted that the sodium combined with glycine had the same effect upon the activity coefficient of the hydroxyl ion as that present as sodium hydroxide.

These measurements indicate that the effect of the salts of amino acids upon the activity of hydroxyl ions is similar to that of the salts of other acids, though perhaps not so great. It is conceivable that the salts of proteins might behave in a more complicated manner. It is possible that although the base bound by amino acids is free, that bound

by protein is not; perhaps because of their colloidal dimensions, and slow mobility. In that case the method of calculating base-binding capacity proposed by Tague, although not applicable to the amino acids, for which it was proposed, would still apply in the case of the proteins. Evidence, derived from the base-binding capacity of different amounts of casein, and also of certain other proteins, renders this view improbable.

The simplest method of calculating the base-binding capacity of proteins from electromotive force measurements is presented by the relation[12]

$$pNaOH = pOH^- - p\gamma \qquad (7)$$

For if one assumes that the activity coefficient depends only upon the total sodium, in an alkaline solution, the free sodium hydroxide can be directly estimated from the hydroxyl potential. This procedure we have tentatively adopted. It occurred to us, however, that the high valence of protein anions might further depress the activity. Accordingly we studied four bivalent acids, sulfuric, oxalic, glutamic, and aspartic, the two latter amino acids. One of these was also studied by Tague. The results with these acids are also included in Table III. They suggest that anions of higher valence have greater effects upon the activity of the hydroxyl ion. This effect might be expected to increase with the ionic strength (21, 29), but the effect of the amino acids studied at this concentration was not so great as that of other divalent salts. This effect is being investigated further. The only conclusion that we feel free to draw from these preliminary data is that the base bound by amino acids in alkaline solutions depresses the activity of the hydroxyl ions.

Because of their high valence, protein anions may, of course, further depress the activity of the hydroxyl ion. Uncertainty regarding the valence type to be ascribed to proteins makes it difficult, however, to apply a correction for this factor,[13] and their very low concentration may make it unnecessary. Accordingly we have tentatively used the

[12] An equation depending upon the same theoretical principle has been employed by Pauli (3), p. 94.

[13] It should be pointed out that this correction is in the opposite sense from that involved in Tague's method.

values of γ deduced from measurements of sodium hydroxide solutions in the calculation of our data.

Long before the Tague method of calculating base-binding had been suggested, Robertson (14) employed a method analogous to ours. He determined the base bound by casein and several other proteins by measuring the electromotive force between two hydrogen electrodes; one containing a sodium hydroxide solution in which casein was dissolved, and the other a sodium hydroxide solution of the same concentration. We have made a number of measurements of this kind, and report the results in Table IV. The electromotive force set up in this way (e) is equal to the difference between the electromotive force of the sodium hydroxide solution against 0.1 N calomel (b or c) and the protein containing sodium hydroxide against the calomel half cell (d). Since it is far more convenient always to refer measurements to the same standard, and since calomel electrodes are far more reproducible than sodium hydroxide half cells, we have not adopted Robertson's procedure. It rests upon the same theoretical assumptions as our own method, however, and is capable of giving similar results.

V.

The Effect of Time upon the Base-Combining Capacity of Casein.

Proteins hydrolyze even in weakly alkaline solutions, such as those we have employed in measuring the base-binding capacity of casein. During hydrolysis an increased number of acid groups becomes free and the base-binding capacity therefore increases.

In our experiments the base-binding capacity of casein always increased with time, and increased more rapidly the more alkaline the solution. In order to establish the fact that this increase in base-binding capacity was due to hydrolysis, and not only to a gradual attainment of equilibrium, we have had recourse to other criteria of hydrolysis than base-binding.

When casein is hydrolyzed by alkali an albumose, called caseose, is one of the early decomposition products. Dakin has noted the behavior of this substance in connection with his studies of the racemization of casein (30). It is relatively soluble in water in the range in which casein is only very slightly soluble. It is, however, precipitated from

TABLE IV.

The Calculation of Base-Combining Capacity.

Preparation and experiment No.	NaOH concentration.	Temperature.	E.M.F. of NaOH against 0.1 N calomel.		E.M.F. of NaOH containing 10 gm. of casein per liter against 0.1 N calomel.	E.M.F. of NaOH against the same concentration of NaOH containing 10 gm. of casein per liter.		NaOH bound by 10 gm. of casein.	
	(*a*)		(*b*) Calculated.	(*c*) Observed.	(*d*) Observed.	(*e*) Observed.	(*b* − *d*) Calculated.	Calculated by Robertson's method from (*a*) and (*e*).	Calculated by our method from (*b*) and (*d*).
	N.	°C.	*volts*	*volts*	*volts*	*volts*	*volts*	*mols*	*mols*
VI 88	0.025	20.6	1.0573	1.0588	1.0349	0.0238	0.0224	0.0153	0.0146
		20.2	1.0571	1.0588	1.0319	0.0268	0.0252	0.0163	0.0158
		20.6	1.0573	1.0590	1.0342	0.0245	0.0231	0.0155	0.0149
		19.8	1.0570	1.0590	1.0329	0.0259	0.0241	0.0160	0.0154
86	0.030	22.0	1.0622	1.0636	1.0434	0.0203	0.0188	0.0165	0.0157
		20.1	1.0614		1.0412		0.0202		0.0166
		22.0	1.0622		1.0449		0.0173		0.0148
87	0.040	19.7	1.0682	1.0696	1.0559	0.0138	0.0123	0.0168	0.0155
		19.9	1.0683	1.0696	1.0568	0.0126	0.0115	0.0157	0.0147
		20.2	1.0684	1.0704	1.0549	0.0164	0.0135	0.0191	0.0166
		21.3	1.0689		1.0567	0.0132	0.0122	0.0162	0.0153
							Average..	0.0164	0.0154
XIII 76	0.025	21.7	1.0576	1.0588	1.0366	0.0224	0.0210	0.0147	0.0141
		23.2	1.0582	1.0597	(1.0408)	0.0224	(0.0174)	0.0146	(0.0123)
79		23.4	1.0583	1.0594	1.0379	0.0225	0.0204	0.0146	0.0137
75	0.030	20.0	1.0614	1.0610	1.0446	0.0151	0.0168	0.0135	0.0146
		20.5	1.0616	1.0611	1.0440		0.0176		0.0150
78		23.6	1.0629	1.0625	1.0467	0.0161	0.0162	0.0141	0.0139
		23.3	1.0628	1.0622	1.0455	0.0169	0.0173	0.0145	0.0147
77	0.040	22.9	1.0696	1.0693	1.0595	0.0100	0.0101	0.0130	0.0130
		23.2	1.0697	1.0701	1.0589	0.0117	0.0108	0.0147	0.0136
80		23.4	1.0698	1.0696	1.0594	0.0103	0.0104	0.0134	0.0132
							Average..	0.0141	0.0138

a half saturated solution of ammonium sulfate. In the experiments tabulated below we have therefore correlated the change in base-binding capacity with the appearance of caseose. Caseose has been demonstrated by two methods. In both, aliquot parts of the casein that had been dissolved in sodium hydroxide were neutralized, from time to time, by adding an amount of hydrochloric acid equivalent to the sodium hydroxide they contained. A small amount of an acetate mixture of pH 4.7 was then added to ensure complete coagulation of the casein at its isoelectric point. After equilibrium had been established in the manner we have already described (31) the precipitated casein was removed by filtration and nitrogen determinations made upon the filtrate. Starting with values for solubility of the order of those of of casein in water[14] these gradually increased with increase in base-binding capacity. In order to demonstrate that this increase in soluble nitrogenous material was due to caseose we half saturated aliquot parts of the filtrate with ammonium sulfate. Whereas no precipitate appeared in the filtrates from casein that had been in alkali for but a short time, the caseose content increased with time. This is indicated not only by the nitrogen determinations but by the density of the precipitate in half saturated ammonium sulfate.

It is evident that these experiments offer a quantitative method of studying hydrolysis. For the present we have made no effort to refine the measurements, and have merely used the method to determine how long we could leave casein in alkaline solutions before this type of hydrolysis would interfere with our results. These experiments indicate that hydrolysis of casein, leading to the formation of caseose, introduces no error greater than that involved in measuring the base-bound, provided the measurements are made within the first 10 hours. The fact that the base-binding capacity of each casein preparation was at first identical in different concentrations of sodium hydroxide, although the rates of hydrolysis in these solutions were different, leads us to believe that equilibrium was attained between the casein and the alkali before hydrolysis was measurable.

[14] The solubility was slightly greater owing to the solvent action of the salt present (32, 33).

TABLE V.

Hydrolysis of Casein Dissolved in Sodium Hydroxide.

Casein preparation.	Experiment No.	NaOH concentration.	Length of time in NaOH.	NaOH bound by 10 gm. of casein.	Solubility at isoelectric point.	Caseose formed.
					Mg. of N in 25 cc. of filtrate.	Protease soluble in H_2O at pH 4.7 but insoluble in 0.5 saturated $(NH_4)_2SO_4$.
		N.	*hrs.*	*mols*		
XIII	76	0.025	½	0.0141	0.63	0
	79		½	0.0137	0.68	0
	76		6	(0.0123)	0.63	?
	79		19	(0.0129)	0.76	+
	79		23	0.0153	0.80	+
	76		25	(0.0115)	0.69	+
	79		46	0.0138	0.70	++
	76		49	0.0149	(0.65)	++
	79		68	0.0151	0.80	+++
	76		75	0.0161	0.88	+++
	75	0.030	½	0.0146	0.55	?
	78		1	0.0139	0.58	0
	75		4	0.0150	0.70	+
	78		4	0.0147	0.58	++
	78		22	0.0153	0.60	+++
	75		23	0.0160	0.65	++
	78		71	0.0167	1.05	++++
	75		76	(0.0143)	(0.83)	++
	75		101	0.0176	1.04	++++
	78		118	0.0175	1.01	++++
	75		149	0.0193		
	80	0.040	½	0.0132	0.78	
	77		1	0.0130	0.63	0
	77		5	0.0136	0.65	?
	80		19	(0.0117)	0.80	+++
	80		24	0.0135	0.81	+++
	77		28	0.0149	0.65	+
	80		44	0.0153	1.00	++++
	77		49	0.0163	0.88	++
	80		68	0.0175	1.20	
	77		95	0.0182	1.05	+++++

VI.

The Base-Combining Capacity of Casein.

The base-combining capacities of ten different casein[15] preparations have been studied. The amount of casein has been somewhat varied and the concentration of sodium hydroxide increased from 0.025 to 0.050 N. Although the sodium hydroxide combining capacity varied slightly from preparation to preparation, in every preparation the base combined by the protein was independent of the concentration of free base in the systems here reported. Doubling the concentration of total base, which often involved trebling, and very nearly quadrupling the concentration of free base, resulted in no increase in the base bound by the casein. Accordingly we must conclude that this neutralization reaction was chemical in nature, depending upon the dissociation of acid groups in the casein molecule.

The first six casein preparations that were studied in this way were prepared by a method that has elsewhere been described in detail (31). In our practise,

"After the casein has been precipitated at its isoelectric point and washed with distilled water according to the method of Baker and Van Slyke, only enough sodium hydroxide was added to bring the casein to a neutral reaction. In this our method differed from that of Hammarsten in which enough sodium hydroxide is added to dissolve completely the casein. Loeb's observation suggests that the casein that persists at a neutral reaction is largely combined with divalent bases. We have accordingly removed and discarded this precipitate either by centrifugation (in a Sharples centrifuge at nearly 30,000 revolutions a minute) or by filtration through filter paper pulp. The casein in the filtrate or in the centrifugate was then reprecipitated at its isoelectric point by the addition of hydrochloric acid, washed, and again dissolved by sodium hydroxide. It has been our experience that the casein completely dissolved the second time at a neutral reaction. The casein in this clear neutral solution was finally precipitated and purified at the isoelectric point."

The original purpose in adding "only enough sodium hydroxide . . . . to bring the casein to a neutral reaction" was to discard the fraction that dissolved only at relatively great alkalinities. The results of the measurements of base-binding capacity indicate that this

[15] All were prepared from H. P. Hood and Sons pasteurized milk. The effects of pasteurization will subsequently be considered.

TABLE VI.

Maximum Base-Combining Capacity of Casein.

Preparation and experiment No.	Casein in 1 liter.	Concentration of NaOH.	E.M.F.	Temperature.	pOH^-	pNaOH ($pOH^- - p_Y$)	Concentration of free NaOH.	NaOH bound.	NaOH bound by 1 gm. of casein.
	gm.	N.	*volts*	°C.			N.	*mols*	*mols*
VI 88	10.00	0.025	1.0349	20.6	2.040	1.985	0.0104	0.0146	0.00146
			1.0319	20.2	2.092	2.037	0.0092	0.0158	0.00158
			1.0342	20.6	2.052	1.997	0.0101	0.0149	0.00149
			1.0329	19.8	2.072	2.017	0.0096	0.0154	0.00144
86	10.00	0.030	1.0434	22.0	1.904	1.845	0.0143	0.0157	0.00157
			1.0412	20.1	1.932	1.873	0.0134	0.0166	0.00166
			1.0449	22.0	1.878	1.819	0.0152	0.0148	0.00148
87	10.00	0.040	1.0559	19.7	1.675	1.610	0.0245	0.0155	0.00155
			1.0568	19.9	1.662	1.597	0.0253	0.0147	0.00147
			1.0549	20.2	1.696	1.631	0.0234	0.0166	0.00166
			1.0567	21.3	1.672	1.607	0.0247	0.0153	0.00153
								Average..	0.00154
XII 97	10.00	0.025	1.0330	20.8	2.074	2.019	0.0096	0.0154	0.00154
			1.0333	19.8	2.065	2.010	0.0098	0.0152	0.00152
			1.0320	19.3	2.085	2.030	0.0093	0.0157	0.00157
			1.0313	19.8	2.099	2.044	0.0091	0.0160	0.00160
96	10.00	0.030	1.0431	21.4	1.905	1.846	0.0143	0.0157	0.00157
			1.0430	21.4	1.907	1.848	0.0142	0.0158	0.00158
			1.0410	18.7	1.924	1.865	0.0136	0.0164	0.00164
			1.0412	18.7	1.921	1.862	0.0138	0.0162	0.00162
98	10.00	0.040	1.0560	19.4	1.672	1.607	0.0247	0.0153	0.00153
			1.0551	19.4	1.686	1.621	0.0239	0.0161	0.00161
			1.0554	19.2	1.680	1.615	0.0243	0.0157	0.00157
			1.0568	19.2	1.656	1.591	0.0256	0.0144	0.00144
105	10.00	0.040	1.0557	20.4	1.683	1.618	0.0241	0.0159	0.00159
			1.0558	20.4	1.682	1.617	0.0242	0.0158	0.00158
			1.0558	21.5	1.689	1.624	0.0238	0.0162	0.00162
			1.0559	21.0	1.683	1.618	0.0241	0.0159	0.00159
108	10.00	0.050	1.0643	21.2	1.540	1.469	0.0340	0.0160	0.00160
			1.0646	21.3	1.536	1.465	0.0343	0.0157	0.00157
			1.0651	22.0	1.533	1.462	0.0345	0.0155	0.00155
			1.0648	21.8	1.537	1.466	0.0342	0.0158	0.00158

TABLE VI—*Continued.*

Preparation and experiment No.	Casein in 1 liter.	Concentration of NaOH.	E.M.F.	Temperature.	pOH⁻	pNaOH (pOH⁻ − pγ)	Concentration of free NaOH.	NaOH bound.	NaOH bound by 1 gm. of casein.
	gm.	*N.*	*volts*	*°C.*			*N.*	*mols*	*mols*
XII 109	10.00	0.050	1.0649	21.2	1.530	1.459	0.0348	0.0152	0.00152
			1.0654	21.9	1.527	1.456	0.0350	0.0150	0.00150
			1.0657	21.7	1.521	1.450	0.0355	0.0145	0.00145
			1.0652	21.5	1.528	1.457	0.0349	0.0151	0.00151
								Average..	0.00156
XIII 76	10.00	0.025	1.0366	21.7	2.019	1.964	0.0109	0.0141	0.00141
			1.0408	23.2	1.951	1.896	0.0127	0.0123	0.00123
79	10.00	0.025	1.0379	23.4	2.002	1.947	0.0113	0.0137	0.00137
75	10.00	0.030	1.0446	20.0	1.872	1.813	0.0154	0.0146	0.00146
			1.0440	20.5	1.884	1.825	0.0150	0.0150	0.00150
78	10.00	0.030	1.0467	23.6	1.853	1.794	0.0161	0.0139	0.00139
			1.0455	23.3	1.873	1.814	0.0153	0.0147	0.00147
77	10.00	0.040	1.0595	22.9	1.634	1.569	0.0270	0.0130	0.00130
			1.0589	23.2	1.643	1.578	0.0264	0.0136	0.00136
80	10.00	0.040	1.0594	23.4	1.637	1.572	0.0268	0.0132	0.00132
106	10.00	0.050	1.0660	20.8	1.508	1.437	0.0366	0.0134	0.00134
			1.0683	21.1	1.506	1.435	0.0367	0.0133	0.00133
			1.0666	21.9	1.507	1.436	0.0366	0.0134	0.00134
			1.0669	21.8	1.501	1.430	0.0372	0.0128	0.00128
								Average..	0.00136
XIV 99	10.00	0.025	1.0272	19.6	2.169	2.114	0.0077	0.0173	0.00173
			1.0290	20.8	2.143	2.088	0.0082	0.0168	0.00168
			1.0290	20.8	2.143	2.088	0.0082	0.0168	0.00168
110	9.04	0.025	1.0338	20.4	2.060	2.005	0.0099	0.0151	0.00167
			1.0337	20.4	2.061	2.006	0.0099	0.0151	0.00167
			1.0327	19.9	2.076	2.021	0.0095	0.0155	0.00171
100	10.00	0.030	1.0413	20.4	1.931	1.872	0.0134	0.0166	0.00166
			1.0414	20.7	1.930	1.871	0.0136	0.0165	0.00165

TABLE VI—*Continued.*

Preparation and experiment No.	Casein in 1 liter.	Concentration of NaOH.	E.M.F.	Temperature.	pOH^-	pNaOH ($pOH^- - p_Y$)	Concentration of free NaOH.	NaOH bound.	NaOH bound by 1 gm. of casein.
	gm.	N.	*volts*	°C.			N.	*mols*	*mols*
XIV 111	9.04	0.030	1.0438	20.5	1.887	1.828	0.0149	0.0151	0.00167
			1.0431	20.4	1.900	1.841	0.0144	0.0156	0.00173
			1.0428	20.6	1.905	1.846	0.0143	0.0157	0.00174
119	9.04	0.030	1.0444	22.8	1.890	1.831	0.0148	0.0152	0.00168
			1.0452	23.1	1.878	1.819	0.0152	0.0148	0.00164
			1.0450	23.0	1.881	1.822	0.0151	0.0149	0.00165
101	10.00	0.040	1.0540	20.9	1.715	1.650	0.0224	0.0176	0.00176
			1.0552	21.5	1.699	1.634	0.0232	0.0168	0.00168
			1.0551	22.6	1.706	1.641	0.0229	0.0171	0.00171
			1.0549	22.2	1.709	1.644	0.0227	0.0173	0.00173
112	9.04	0.040	1.0576	21.6	1.658	1.593	0.0255	0.0145	0.00160
			1.0579	22.4	1.659	1.594	0.0255	0.0145	0.00160
			1.0567	21.2	1.670	1.605	0.0248	0.0152	0.00168
120	9.04	0.040	1.0577	22.8	1.663	1.598	0.0252	0.0148	0.00164
			1.0579	23.2	1.660	1.595	0.0254	0.0146	0.00162
			1.0571	22.7	1.673	1.608	0.0247	0.0153	0.00169
107	10.00	0.050	1.0639	21.1	1.547	1.476	0.0334	0.0166	0.00166
			1.0646	22.1	1.543	1.472	0.0337	0.0163	0.00163
			1.0643	21.0	1.539	1.468	0.0340	0.0160	0.00160
113	9.04	0.050	1.0653	20.7	1.520	1.449	0.0356	0.0144	0.00159
			1.0659	21.8	1.518	1.447	0.0357	0.0143	0.00158
			1.0659	22.1	1.520	1.449	0.0356	0.0144	0.00159
								Average..	0.00166
XV 114	7.134	0.025	1.0423	20.4	1.914	1.859	0.0138	0.0112	0.00157
			1.0424	21.2	1.915	1.860	0.0138	0.0112	0.00157
			1.0434	21.8	1.902	1.847	0.0142	0.0108	0.00151
			1.0420	21.4	1.924	1.869	0.0135	0.0115	0.00161
			1.0424	19.8	1.908	1.853	0.0140	0.0110	0.00154
115	7.134	0.030	1.0501	20.3	1.779	1.720	0.0191	0.0109	0.00153
			1.0497	21.7	1.795	1.736	0.0184	0.0116	0.00163
			1.0507	21.6	1.776	1.717	0.0192	0.0108	0.00151
			1.0504	21.8	1.783	1.724	0.0189	0.0111	0.00156
			1.0504	19.5	1.769	1.710	0.0195	0.0105	0.00147
			1.0488	19.8	1.798	1.739	0.0182	0.0118	0.00165

TABLE VI—*Concluded.*

Preparation and experiment No.	Casein in 1 liter.	Concentration of NaOH.	E.M.F.	Temperature.	pOH⁻	pNaOH (pOH⁻ − py)	Concentration of free NaOH.	NaOH bound.	NaOH bound by 1 gm. of casein.
	gm.	N.	*volts*	°C.			N.	*mols*	*mols*
XV 116	7.134	0.040	1.0614	21.7	1.595	1.530	0.0295	0.0105	0.00147
			1.0612	21.4	1.595	1.530	0.0295	0.0105	0.00147
			1.0613	20.8	1.589	1.524	0.0299	0.0101	0.00142
			1.0614	21.6	1.593	1.528	0.0296	0.0104	0.00146
			1.0612	21.2	1.593	1.528	0.0296	0.0104	0.00146
			1.0613	21.2	1.592	1.527	0.0297	0.0103	0.00144
117	7.134	0.050	1.0671	21.0	1.491	1.420	0.0380	0.0120	0.00168
			1.0670	21.0	1.493	1.422	0.0378	0.0122	0.00171
			1.0675	21.1	1.486	1.415	0.0385	0.0115	0.00161
			1.0672	21.0	1.489	1.418	0.0382	0.0118	0.00165
			1.0671	21.0	1.491	1.420	0.0380	0.0120	0.00168
118	10.00	0.050	1.0640	20.3	1.540	1.469	0.0340	0.0160	0.00160
			1.0641	20.5	1.539	1.468	0.0340	0.0160	0.00160
			1.0641	20.6	1.539	1.468	0.0340	0.0160	0.00160
			1.0641	20.6	1.539	1.468	0.0340	0.0160	0.00160
								Average..	0.00156
XVIa 124	10.84	0.03	1.0422	20.3	1.915	1.856	0.0139	0.0161	0.00149
			1.0437	20.1	1.889	1.830	0.0148	0.0152	0.00140
			1.0431	19.7	1.895	1.836	0.0146	0.0154	0.00142
			1.0431	19.6	1.895	1.836	0.0146	0.0154	0.00142
127	11.34	0.04	1.0562	19.6	1.669	1.604	0.0249	0.0151	0.00133
			1.0557	20.5	1.683	1.618	0.0241	0.0159	0.00140
			1.0552	20.2	1.691	1.626	0.0237	0.0163	0.00144
			1.0549	20.0	1.694	1.629	0.0235	0.0165	0.00146
			1.0555	19.7	1.682	1.617	0.0242	0.0158	0.00139
126	11.54	0.05	1.0632	20.4	1.554	1.483	0.0329	0.0171	0.00148
			1.0642	20.5	1.537	1.466	0.0342	0.0158	0.00137
			1.0646	20.5	1.531	1.460	0.0347	0.0153	0.00133
			1.0645	20.5	1.532	1.461	0.0346	0.0154	0.00133
			1.0638	20.0	1.541	1.470	0.0339	0.0161	0.00140
								Average..	0.00140

practise also served another purpose; for casein that had been brought to an alkaline reaction acquired a greater capacity to combine with base than that which had not been so treated.

The electromotive force measurements upon sodium hydroxide solutions containing casein, from which these base-combining capacities have been calculated, are recorded in Table VI. The method of calculation has already been described. The results are given in detail so that they may be available for recalculation should a method of determining the effect of casein upon the activity of the hydroxyl ion be subsequently discovered. From the electromotive force measurements and the temperature in the fourth and fifth columns the pOH^- has been calculated by means of equations (1) and (2). From the hydroxyl potential and equation (7) the negative logarithm of the sodium hydroxide concentration has been estimated (Column 7); and from this the concentration of free sodium hydroxide (Column 8). The difference between the concentration of free sodium hydroxide, and the concentration of total sodium hydroxide in the system (Column 3) yields the base bound by the casein (Column 9). From this quantity, and the amount of casein in the system (Column 2) the amount of base bound by 1 gm. of casein has been calculated (Column 10). The amount of casein in these systems has been estimated from nitrogen determinations, on the assumption that 1 gm. of casein contains 0.156 gm. of nitrogen (34).

Of the first six casein preparations studied, five showed low, but not identical base-binding capacities. The exception, Preparation XIV, had a slightly higher base-binding capacity than the others, but a lower base-binding capacity, as we shall presently show, than casein prepared by Hammarsten's method. Of the other five, Preparations XIII and XVIa bound the smallest amounts of base, while preparations VI, XII, and XV bound slightly greater, but almost identical, amounts. The averages of the results obtained with these different preparations have been arranged, in Table VII, in the order of their base-combining capacities. From the amount of base bound by each gram of casein the equivalent weight of casein combined with each mol of base has been calculated. On the assumption that the basic equivalents represent acid groups, the number of such groups that casein contains, provided its molecular weight is considered as 12,800, has also been calculated.

The difference in base-binding capacity of these different preparations was far greater than the experimental error. All of the electromotive force measurements made on each preparation—usually with different electrodes—are recorded in Table VI. From them it may be seen that the highest base-binding observed with either Preparation XIII or XVIa was 0.00150 mols of sodium hydroxide to 1 gm. of casein, or a value lower than the average for preparations VI, XII, and XV. Similarly the lowest value observed with these preparations was higher than the average of XIII or XVIa. We must therefore admit that the base-combining capacities of these different preparations were not identical, and seek further for an explanation of the differences observed.

TABLE VII.

Casein preparation.	NaOH bound by 1 gm. of casein.	Equivalent weight of casein bound by 1 mol of NaOH.	No. of assumed casein equivalents.	Calculated minimal molecular weight.
	mols	*gm.*		
XIII	0.00136	735	17	12,495
XVIa	0.00140	714	18	12,852
VI	0.00154	649	20	12,980
XII	0.00156	641	20	12,820
XV	0.00156	641	20	12,820
XIV	0.00166	602	21	12,642

The results obtained with these six preparations do indicate, however, the general magnitude of the base bound by 1 gm. of casein. The equivalent weight of casein bound by each mol of base varied between 600 and 735 gm. On the basis of a minimal molecular weight of 12,800, casein must, therefore, contain between seventeen and twenty-one acid groups. The smaller number of groups can be exactly accounted for on the basis of the free carboxyl groups in casein, and the larger number might be expected if the phosphorus in casein is considered as phosphoric acid, and the acid groups of this acid are added to the free carboxyl groups. From a consideration of the composition of casein we came to the conclusion that this protein might contain from nineteen to twenty-eight acid groups and that 1 gm. should therefore combine with from 148.7 to 217.4 $\times$ 10^{-5} mols of sodium hydroxide. The results obtained by measuring the base-

binding capacity by physicochemical means are well within these limits. We must therefore conclude that the free acid groups that we know exist in the casein molecule are of the order demanded to account for its capacity to combine with base.

VII.

The Effect of the Method of Preparation upon the Base-Combining Capacity of Casein.

Robertson measured the base-combining capacity of casein in 1910. The method that he employed in making these measurements has

TABLE VIII.

Maximum Base-Combining Capacity of Casein.
Robertson (14).

Casein in 1 liter.	Concentration of NaOH.	Concentration of NaOH bound.	NaOH bound by 1 gm. of casein.
gm.	N.	N.	*mols*
5.0	0.02000	0.00917	0.001834
10.0	0.02907	0.01886	0.001886
10.0	0.03028	0.01815	0.001815
10.0	0.02500	0.01748	0.001748
10.0	0.0300	0.01763	0.001763
20.0	0.0500	0.03478	0.001739
			Average..0.001797

already been considered. "It rests upon the same theoretical assumptions as our own method . . . . and is capable of giving similar results." Yet Robertson found that 1 gm. of casein combined approximately 0.0018 mols of potassium hydroxide; a higher value than we observed with any of the previously considered preparations. The six measurements of Robertson in which the casein was saturated with base are tabulated above (Table VIII). His results are in good agreement with each other, and unquestionably represent the base-combining capacity of the casein preparation that he employed.

In his investigations Robertson "employed the C.P. casein *nach Hammarsten.*"[16] In the method of preparing casein introduced by Hammarsten the protein is exposed to greater concentrations of alkali than in the method of Van Slyke and Baker (35), or in the modification of their method that we have employed (31). In order to determine whether this treatment increased the base-combining capacity of casein

TABLE IX.

Maximum Base-Combining Capacity of Casein Prepared by Hammarsten's Method.

Preparation and experiment No.	Casein in 1 liter.	Concentration of NaOH.	E.M.F.	Temperature.	pOH^-	pNaOH ($pOH^- - p_\gamma$)	Concentration of free NaOH.	NaOH bound.	NaOH bound by 1 gm. of casein.
	gm.	N.	*volts*	°C.			N.	*mols*	*mols*
H 148	9.30	0.03	1.0432	23.0	1.912	1.853	0.0140	0.0160	0.00172
			1.0430	23.0	1.916	1.857	0.0139	0.0161	0.00173
			1.0416	23.0	1.940	1.881	0.0132	0.0168	0.00181
			1.0425	23.2	1.923	1.864	0.0137	0.0163	0.00175
150	9.68	0.04	1.0566	23.0	1.684	1.619	0.0240	0.0160	0.00165
			1.0559	22.6	1.693	1.628	0.0236	0.0164	0.00169
			1.0532	22.8	1.740	1.675	0.0211	0.0189	0.00195
			1.0564	23.1	1.688	1.623	0.0238	0.0162	0.00167
149	8.30	0.05	1.0661	22.9	1.523	1.452	0.0353	0.0147	0.00177
			1.0660	22.5	1.521	1.450	0.0355	0.0145	0.00175
			1.0660	23.0	1.524	1.453	0.0352	0.0148	0.00178
			1.0650	22.5	1.539	1.468	0.0340	0.0160	0.00193
								Average..	0.00177

we procured a Kahlbaum preparation of casein *nach Hammarsten* that had been in the possession of the Department of Biological Chemistry since before the war. This casein was repeatedly triturated with water, and was finally studied in precisely the same way as the other preparations. The results are reported in Table IX. The average base-

[16] For a detailed description of the further methods that Robertson employed in purifying his casein see Robertson (4), p. 39.

Pauli and Kryz ((3), p. 106), also employed casein "*nach Hammarsten.*" Their experiments indicate a higher base-combining capacity for casein than either Robertson or we have obtained.

combining capacity was 0.00177 mols of sodium hydroxide per gm. of casein, or almost exactly that found by Robertson.

In connection with studies of the strength of the dissociation of the acid groups in casein, that will elsewhere be reported in detail, we have been forced to the conclusion that certain of the acid "valencies combined with base very slowly, as though they were internally bound" (1).[17] It therefore occurred to us that these groups were responsible for the greater base-binding capacity of casein prepared *nach Hammarsten.*

In order to test this hypothesis the next two preparations, XVIII and XIX, were brought to alkaline reactions when they were first redissolved, and were then treated in much the same manner as the earlier preparations. This procedure was not satisfactory, largely because of the length of time that the casein remained dissolved in alkaline solution. As a result the casein solutions acquired a brownish color, and some caseose formed. The caseose was largely removed by reprecipitating and redissolving the preparations at a neutral reaction, but the brownish color persisted. We are therefore not inclined to place much confidence in the results that have been obtained with these two preparations. They are reported in Table X, and yielded the highest base-combining capacity that has been noted, even higher than casein prepared *nach Hammarsten.*

The effect of the alkalinity reached in the course of purification was more systematically studied in Casein XX. This casein was first redissolved at a neutral reaction, and then reprecipitated, in precisely the same manner as the earlier preparations. Like them it was then redissolved at a neutral reaction. This solution was then divided into four parts, which were brought to different alkalinities by the addition of different amounts of sodium hydroxide. Thus Fractions (a) and (b) although less alkaline than casein *nach Hammarsten*, were made more alkaline than our ordinary preparations; Fraction (c) was brought to approximately the reaction reached by casein *nach Hammarsten*; and enough alkali was added to Fraction (d) to completely combine with the acid groups in the casein molecule. None of the fractions was allowed to remain at these alkalinities for less than

[17] Cohn (1), p. 92.

TABLE X.

Maximum Base-Combining Capacity of Casein.

Preparation and experiment No.	Casein in 1 liter.	Concentration of NaOH.	E.M.F.	Temperature.	pOH⁻	pNaOH (pOH⁻ − py)	Concentration of free NaOH.	NaOH bound.	NaOH bound by 1 gm. of casein.
	gm.	N.	*volts*	°C.			N.	*mols*	*mols*
XVIIIb 195	13.02	0.030	1.0204	20.2	2.289	2.230	0.0059	0.0241	0.00185
			1.0191	20.1	2.312	2.253	0.0056	0.0244	0.00187
			1.0185	20.2	2.322	2.263	0.0055	0.0245	0.00188
			1.0207	20.6	2.284	2.225	0.0060	0.0240	0.00184
207	12.40	0.030	1.0250	22.0	2.218	2.159	0.0069	0.0231	0.00186
			1.0240	22.0	2.235	2.176	0.0067	0.0233	0.00188
			1.0238	22.4	2.241	2.182	0.0066	0.0234	0.00189
			1.0251	22.5	2.217	2.158	0.0070	0.0230	0.00186
208	12.40	0.040	1.0479	22.2	1.827	1.762	0.0173	0.0227	0.00183
			1.0466	22.1	1.850	1.785	0.0164	0.0236	0.00190
			1.0469	22.0	1.844	1.779	0.0166	0.0234	0.00189
			1.0473	22.5	1.839	1.774	0.0168	0.0232	0.00187
197	21.02	0.040	0.9962	19.5	2.703	2.638	0.0023	0.0377	0.00179
			0.9976	19.7	2.679	2.614	0.0024	0.0376	0.00179
			0.9976	20.3	2.682	2.617	0.0024	0.0376	0.00179
196	21.66	0.050	1.0253	19.6	2.201	2.130	0.0074	0.0426	0.00197
			1.0243	20.1	2.222	2.151	0.0071	0.0429	0.00198
			1.0259	20.2	2.195	2.124	0.0075	0.0425	0.00196
			1.0240	20.0	2.226	2.155	0.0070	0.0430	0.00199
XIXb 209	9.72	0.030	1.0400	22.3	1.964	1.905	0.0124	0.0176	0.00181
			1.0390	22.3	1.981	1.922	0.0120	0.0180	0.00185
			1.0390	22.2	1.980	1.921	0.0120	0.0180	0.00185
			1.0396	22.7	1.972	1.913	0.0122	0.0178	0.00183
194	16.64	0.040	1.0266	19.6	2.179	2.114	0.0077	0.0323	0.00194
			1.0255	19.8	2.199	2.134	0.0074	0.0326	0.00196
			1.0268	20.1	2.179	2.114	0.0077	0.0323	0.00194
193	16.64	0.050	1.0475	20.0	1.822	1.751	0.0177	0.0323	0.00194
			1.0469	19.9	1.832	1.761	0.0173	0.0327	0.00197
			1.0459	19.8	1.848	1.777	0.0167	0.0333	0.00200
			1.0468	20.7	1.837	1.766	0.0171	0.0329	0.00198

TABLE X—*Continued.*

Preparation and experiment No.	Casein in 1 liter.	Concentration of NaOH.	E.M.F.	Temperature.	pOH⁻	pNaOH (pOH⁻ − pγ)	Concentration of free NaOH.	NaOH bound.	NaOH bound by 1 gm. of casein.
	gm.	*N.*	*volts*	*°C.*			*N.*	*mols*	*mols*
XXa 180	8.24	0.05	1.0642	20.0	1.535	1.464	0.0344	0.0156	0.00189
			1.0649	20.3	1.525	1.454	0.0352	0.0148	0.00180
			1.0649	20.4	1.525	1.454	0.0352	0.0148	0.00180
			1.0641	20.5	1.539	1.468	0.0340	0.0160	0.00194
181	8.46	0.04	1.0558	20.0	1.679	1.614	0.0243	0.0157	0.00186
			1.0552	20.0	1.689	1.624	0.0238	0.0162	0.00191
			1.0564	20.6	1.671	1.606	0.0248	0.0152	0.00180
			1.0571	20.8	1.661	1.596	0.0254	0.0146	0.00173
182	8.18	0.03	1.0439	21.1	1.890	1.831	0.0148	0.0152	0.00186
			1.0430	21.3	1.906	1.847	0.0142	0.0158	0.00193
			1.0440	21.1	1.888	1.829	0.0148	0.0152	0.00186
			1.0442	21.7	1.889	1.830	0.0148	0.0152	0.00186
								Average..	0.00185
XXb 186	10.76	0.05	1.0608	19.3	1.588	1.517	0.0304	0.0196	0.00182
			1.0613	20.0	1.584	1.513	0.0307	0.0193	0.00179
			1.0616	20.4	1.582	1.511	0.0308	0.0192	0.00178
			1.0604	19.7	1.597	1.526	0.0298	0.0202	0.00188
187	11.00	0.04	1.0499	19.3	1.776	1.711	0.0195	0.0205	0.00186
			1.0512	19.8	1.757	1.692	0.0203	0.0197	0.00179
			1.0516	20.2	1.753	1.688	0.0205	0.0195	0.00177
			1.0521	19.0	1.736	1.671	0.0213	0.0187	0.00170
188	10.76	0.03	1.0351	19.4	2.032	1.973	0.0106	0.0194	0.00180
			1.0342	19.5	2.047	1.988	0.0103	0.0197	0.00183
			1.0356	20.1	2.028	1.969	0.0107	0.0193	0.00179
			1.0355	20.0	2.028	1.969	0.0107	0.0193	0.00179
								Average..	0.00180
XXc 183	10.54	0.05	1.0611	19.0	1.580	1.509	0.0310	0.0190	0.00180
			1.0605	18.9	1.589	1.518	0.0303	0.0197	0.00187
			1.0611	18.9	1.579	1.508	0.0310	0.0190	0.00180
			1.0600	19.2	1.601	1.530	0.0295	0.0205	0.00194

TABLE X—*Concluded.*

Preparation and experiment No.	Casein in 1 liter.	Concentration of NaOH.	E.M.F.	Temperature.	pOH⁻	pNaOH (pOH⁻ − pγ)	Concentration of free NaOH.	NaOH bound.	NaOH bound by 1 gm. of casein.
	gm.	N.	*volts*	°C.			N.	*mols*	*mols*
XXc 184	10.86	0.04	1.0511	18.8	1.750	1.685	0.0207	0.0193	0.00178
			1.0499	19.0	1.774	1.709	0.0195	0.0205	0.00189
			1.0509	19.2	1.758	1.693	0.0203	0.0197	0.00181
			1.0507	18.5	1.754	1.689	0.0204	0.0196	0.00180
185	10.44	0.03	1.0373	21.4	2.004	1.945	0.0114	0.0186	0.00178
			1.0361	21.3	2.025	1.966	0.0108	0.0192	0.00184
			1.0371	21.4	2.008	1.949	0.0112	0.0188	0.00180
			1.0374	21.8	2.005	1.946	0.0113	0.0187	0.00179
								Average..	0.00183
XXd 189	12.62	0.05	1.0579	19.7	1.640	1.569	0.0270	0.0230	0.00182
			1.0576	19.2	1.642	1.571	0.0269	0.0231	0.00183
			1.0567	20.1	1.665	1.594	0.0255	0.0245	0.00194
			1.0578	20.7	1.649	1.578	0.0264	0.0236	0.00187
191	10.58	0.04	1.0518	20.8	1.752	1.687	0.0206	0.0194	0.00183
			1.0505	20.6	1.773	1.708	0.0196	0.0204	0.00193
			1.0519	20.8	1.750	1.685	0.0207	0.0193	0.00182
			1.0514	20.4	1.757	1.692	0.0203	0.0197	0.00186
190	10.42	0.03	1.0372	20.3	2.001	1.942	0.0114	0.0186	0.00179
			1.0355	20.2	2.030	1.971	0.0107	0.0193	0.00185
			1.0368	20.2	2.007	1.948	0.0113	0.0187	0.00179
			1.0373	20.8	2.001	1.942	0.0114	0.0186	0.00179
								Average..	0.00184

½ or more than 3 hours. They were then reprecipitated, washed, once more redissolved at a neutral reaction in order to remove any caseose that might have formed at greater alkalinities, and again reprecipitated and washed. None of these fractions showed the discoloration observed with XVIII and XIX when they were dissolved in alkali (30). The measurements of their base-combining capacity are also recorded in Table X. It will be noted that all four fractions bound exactly the same amount of base. This observation would

seem to indicate that no ordinary alkaline hydrolysis was here involved. On the other hand the base-combining capacity of all the fractions was far greater than that of the earlier preparations studied, and of the same order as that of casein prepared according to the method introduced by Hammarsten. The base-combining capacity of the preparation of casein that Robertson studied was 0.00180; and of the Kahlbaum preparation that we have studied was 0.00177 mols of base per gm. of casein, while the average base-combining capacity of the four fractions of Casein XX was 0.00183 mols. The last value yields the result that the equivalent combining weight for base of casein that has been modified in the course of its preparation was 535 gm. equivalents.

Four times this equivalent combining weight at saturation yields the equivalent combining weight of casein in the neighborhood of its isoelectric point. "The solubility of casein in systems containing the protein and sodium hydroxide" indicated that "each mol of sodium hydroxide combined with less than 2,166 gm. and probably with 2,096 gm. of casein" (13).[18] Four times 535 leads to 2,140 gm. The results of the electromotive force studies that are now reported are therefore in good agreement with the previously reported solubility studies. They indicate, moreover, that in strongly alkaline solutions casein is capable of combining precisely four times as much base as in the neighborhood of its isoelectric point (1).

Since the equivalent combining weight of casein for base, in the neighborhood of its isoelectric point was one-sixth the minimal molecular weight of this protein, its equivalent weight at saturation should be one twenty-fourth the minimal molecular weight. $24 \times 535 = 12{,}840$, or exactly the minimal molecular weight of this protein. On the basis of a minimal molecular weight of 12,800, casein, that has been treated with alkali, must therefore contain twenty-four acid groups. This number is larger than the number of groups that can be accounted for on the basis of the free carboxyl groups in casein

CORRECTION.

On page 75, Vol. vii, No. 1, September 20, 1924, in lines 11, 18, and 27, and on page 77, under No. 4 of the Summary, in line 3, for *535* read *546*.

On page 75, in line 18, for *2,140* read *2,184*; in line 28, on the same page, for *12,840* read *13,104*.

TABLE X—*Concluded.*

Preparation and experiment No.	Casein in 1 liter.	Concentration of NaOH.	E.M.F.	Temperature.	pOH⁻	pNaOH (pOH⁻ − pγ)	Concentration of free NaOH.	NaOH bound.	NaOH bound by 1 gm. of casein.
	gm.	N.	*volts*	°C.			N.	*mols*	*mols*
XXc 184	10.86	0.04	1.0511	18.8	1.750	1.685	0.0207	0.0193	0.00178
			1.0499	19.0	1.774	1.709	0.0195	0.0205	0.00189
			1.0509	19.2	1.758	1.693	0.0203	0.0197	0.00181
			1.0507	18.5	1.754	1.689	0.0204	0.0196	0.00180
185	10.44	0.03	1.0373	21.4	2.004	1.945	0.0114	0.0186	0.00178
			1.0361	21.3	2.025	1.966	0.0108	0.0192	0.00184
			1.0371	21.4	2.008	1.949	0.0112	0.0188	0.00180
			1.0374	21.8	2.005	1.946	0.0113	0.0187	0.00179
								Average..	0.00183
XXd 189	12.62	0.05	1.0579	19.7	1.640	1.569	0.0270	0.0230	0.00182
			1.0576	19.2	1.642	1.571	0.0269	0.0231	0.00183
			1.0567	20.1	1.665	1.594	0.0255	0.0245	0.00194
			1.0578	20.7	1.649	1.578	0.0264	0.0236	0.00187
191	10.58	0.04	1.0518	20.8	1.752	1.687	0.0206	0.0194	0.00183
			1.0505	20.6	1.773	1.708	0.0196	0.0204	0.00193
			1.0519	20.8	1.750	1.685	0.0207	0.0193	0.00182
			1.0514	20.4	1.757	1.692	0.0203	0.0197	0.00186
190	10.42	0.03	1.0372	20.3	2.001	1.942	0.0114	0.0186	0.00179
			1.0355	20.2	2.030	1.971	0.0107	0.0193	0.00185
			1.0368	20.2	2.007	1.948	0.0113	0.0187	0.00179
			1.0373	20.8	2.001	1.942	0.0114	0.0186	0.00179
								Average..	0.00184

½ or more than 3 hours. They were then reprecipitated, washed, once more redissolved at a neutral reaction in order to remove any caseose that might have formed at greater alkalinities, and again reprecipitated and washed. None of these fractions showed the

seem to indicate that no ordinary alkaline hydrolysis was here involved. On the other hand the base-combining capacity of all the fractions was far greater than that of the earlier preparations studied, and of the same order as that of casein prepared according to the method introduced by Hammarsten. The base-combining capacity of the preparation of casein that Robertson studied was 0.00180; and of the Kahlbaum preparation that we have studied was 0.00177 mols of base per gm. of casein, while the average base-combining capacity of the four fractions of Casein XX was 0.00183 mols. The last value yields the result that the equivalent combining weight for base of casein that has been modified in the course of its preparation was 535 gm. equivalents.

Four times this equivalent combining weight at saturation yields the equivalent combining weight of casein in the neighborhood of its isoelectric point. "The solubility of casein in systems containing the protein and sodium hydroxide" indicated that "each mol of sodium hydroxide combined with less than 2,166 gm. and probably with 2,096 gm. of casein" (13).[18] Four times 535 leads to 2,140 gm. The results of the electromotive force studies that are now reported are therefore in good agreement with the previously reported solubility studies. They indicate, moreover, that in strongly alkaline solutions casein is capable of combining precisely four times as much base as in the neighborhood of its isoelectric point (1).

Since the equivalent combining weight of casein for base, in the neighborhood of its isoelectric point was one-sixth the minimal molecular weight of this protein, its equivalent weight at saturation should be one twenty-fourth the minimal molecular weight. $24 \times 535 = 12{,}840$, or exactly the minimal molecular weight of this protein. On the basis of a minimal molecular weight of 12,800, casein, that has been treated with alkali, must therefore contain twenty-four acid groups. This number is larger than the number of groups that can be accounted for on the basis of the free carboxyl groups in casein (Table I). Nineteen acid groups can be accounted for on this basis. To this number might be added the four tyrosine molecules in the casein molecule (28), provided they retain any base-combining capac-

[18] Cohn and Hendry (13), p. 544.

ity when held in polypeptide linkage, or nine groups derived from phosphoric acid, provided the phosphorus in the casein molecule represents phosphate.[19] Finally it must be admitted that internal -COHN- groups may also be involved in the neutralization of base, although their presence is not demanded, as previous investigators believed, in order to account for the maximum base-combining capacity of this protein.

The increased maximum base-combining capacity of casein that has been brought to alkaline reactions in the course of its preparation may be conceived of in at least three different ways. Either partial racemization of the protein may occur, partial hydrolysis, or the opening of the cyclic anhydrides suggested by Dr. Dakin (13).[20] In any case certain groups bound in the casein molecule become free. The nature of these groups is not indicated by the present experiments, but will be considered in a subsequent communication in connection with a study of the dissociation constants of the acid groups in casein (1).

The number of acid groups that was liberated in casein that was dissolved at alkaline reactions in the course of preparation, may however, be calculated from their base-combining capacities. If 0.00183 mols represents the base bound by 1 gm. of modified casein (XX), then at least one-fourth of this base-combining capacity, or 0.00046 mols, was not possessed by the native protein. The remaining three-fourths, or 0.00137 mols of base per gm. of casein, is exactly equal to the lowest base-combining capacities that we have thus far observed. For the average base-combining capacities of casein XIII and XVIa were 0.00136 and 0.00140, respectively. Alkali must therefore increase the number of acid groups in casein by six, or possibly by seven, groups.

Finally we may conclude, as a result of these investigations of the total number of acid groups in casein prepared according to Ham-

[19] If the weakest phosphoric acid group were free in the casein molecule it would not dissociate at the alkalinities that we have reached. In that case only six groups, instead of nine, would be derived from phosphoric acid. These added to nineteen free groups from dicarboxylic amino acids yield in all twenty-five instead of twenty-eight valences; a result which is in even better agreement with our determinations.

[20] Cohn and Hendry (13), p. 547.

marsten's method, that one-fourth are involved in the passage of casein into solution, and one-fourth are bound in the native protein. The further characterization of these groups, and of the remaining group, which possesses one-half of the base-binding capacity, can be completed only by supplementing the information adduced concerning the number of acid groups in casein with information concerning their strength.

SUMMARY.

1. The methods of measuring the base-combining capacities of proteins have been considered, and the constants and corrections that are employed in their calculation have been critically examined.

2. The base-combining capacities of ten casein preparations have been determined. These differed from each other to a far greater extent than can be attributed to the experimental errors involved in their measurement and calculation. The variations were, moreover, systematic in manner, and can be explained as dependent upon the method employed in the preparation of the casein.

3. Casein that had never been exposed to greater alkalinities than those in which it exists in nature combined with approximately 0.0014 mols of sodium hydroxide per gm., while casein prepared *nach Hammarsten*, and casein that was saturated with base during its preparation, combined with approximately 0.0018 mols of sodium hydroxide per gm.

4. 1 mol of sodium hydroxide, therefore, combined with 735 gm. of casein that had not previously been exposed to alkaline reactions, or with 535 gm. of casein that had previously been saturated with base.

5. If the minimal molecular weight of casein, based upon its tryptophane content, is placed at 12,800, the native protein must, therefore, contain approximately eighteen acid groups, and in addition six acid groups that are released in alkaline solutions, and presumably represent internally bound groups. The total base-combining capacity therefore represents that of a substance with a molecular weight of 12,800 and containing twenty-four acid valences.

6. This base-combining capacity is no greater than can be accounted for on the basis of our knowledge of the structure and composition of

casein. On the basis of a molecular weight of 12,800 casein contains at least 19 molecules of glutamic acid, 4 of aspartic, and 8 of hydroxyglutamic acid. If the amino acids in the protein molecule are bound to each other in polypeptide linkage, each of these thirty-one dicarboxylic acids should yield terminal groups. The ammonia in casein suggests that twelve of these groups are bound as amides. As many as nineteen carboxyl groups may, therefore, be free in the protein molecule.

7. Casein contains phosphorus. If this phosphorus represents phosphoric acid, and if we consider that all of the valences of this acid are either themselves free, or that they have liberated carboxyl groups by entering into the structure of the protein molecule, casein should contain nine additional acid groups.

8. Recent analytical results, therefore, indicate that casein contains at least nineteen, and possibly twenty-eight, free acid groups. The physicochemical measurements presented suggest that casein combines with base as though it contained twenty-four acid groups, of which six, or one-fourth, appear to be bound in the native protein. These experimental results are therefore in close agreement with the expectation on the basis of the classical theory of protein structure.

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STUDIES IN THE PHYSICAL CHEMISTRY OF THE PROTEINS.

IV. The Relation between the Composition of Zein and Its Acid and Basic Properties.

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(Accepted for publication, August 4, 1924.)

I.

INTRODUCTION.

It has not often been possible to consider the relation between the composition of proteins and their behavior. This has been due, in part, to the complexity of this class of substances; a complexity often as bewildering as the organizations and symmetries of the biological systems which proteins constitute, and always more intricate than the stereochemistry of the simpler organic compounds of which proteins are, in turn, composed. The large molecular weights of the proteins, and the large number of amino acids that combine with each other to produce these vast molecules give abundant evidence of their structural complexity. The failure to correlate the behavior of proteins with their composition has probably depended less upon this complexity, however, than upon incomplete knowledge of the amino acids they contain.

Recent advances in the methods of isolating and of estimating amino acids have greatly increased our knowledge of the composition of certain proteins. Thus the composition of casein is now 94.2 per cent[1] known (1). As a result of Dakin's new butyl alcohol method

[1] The convention has been followed of referring to the ratio of the weight of the amino acid recovered, to the weight of the protein analyzed as the percentage composition of the protein. The water absorbed by the amino acids during hydrolysis should, of course, be added to the denominator, in order to yield the true percentage composition.

of extracting protein hydrolysates, the yields of dicarboxylic amino acids isolated from casein have been increased from 16.9 to 36.4 per cent (2). In short, the yield of these divalent acids has been more than doubled. The earlier estimates of the number of dicarboxylic acids in casein were so nearly of the same order as those of ammonia as to suggest that most of the carboxyl groups that were not held in polypeptide linkage were bound as amides (3, 4). From these early estimates it has been inferred "that the predominant method of union of proteins with acids and bases is not through the agency of free amino or carboxyl groups" (5, 6, and 7[2]). The increased yields of dicarboxylic amino acids that Dakin has now obtained are, however, as we have elsewhere shown (8), quite sufficient to account for the capacity of this protein to combine with base. As a result it seemed proper to conclude that a relation obtained between the amino acid composition of casein and its acid properties.

Within the year Dakin (9) has completed a new analysis of the amino acids in zein, as a result of which its composition is now better known than that of any other protein. The amino acids that have been recovered from, or estimated in, zein account for 95.3 per cent[1] of its composition. The information yielded by analysis that makes zein almost unique among the proteins is the complete absence of tryptophane and of lysine, and the presence of only a trace of histidine and arginine. Of the three diamino acids, one, lysine, is therefore lacking, and the other two account for less than 3 per cent.

Although so poor in diamino acids, zein is remarkably rich in dicarboxylic acids; especially glutamic acid. More than one in every four, and nearly one in every three of the amino acids in zein is glutamic acid. This great predominance of dicarboxylic amino acids would indicate an extremely acid protein, were it not that zein also is extremely rich in ammonia. The large amount of ammonia nitrogen suggests, however, that a large part of the free groups of the dicarboxylic acids is held in amide linkage (4).

Analyses alone afford us, therefore, concise information regarding the composition of zein. It has been our intention to investigate in how far the behavior of this protein was interpretable in terms of its composition.

[2] Robertson (7), p. 37.

II.

The Minimal Molecular Weight of Zein.

Since each molecule of protein must be considered to contain at least 1 molecule of each of its constituent amino acids the results of analyses may be used in computing the minimal molecular weights of proteins. Thus we have previously used the tryptophane con-

TABLE I.

The Minimal Molecular Weight of Zein Calculated from Its Composition.

Constituents of zein.		Composition of zein.	Weight containing 1 mol.	Composition of zein.	Minimal molecular weight.
		per cent	*gm.*	*mols*	
Histidine	content*	0.8	19,391	1	19,391
Serine	" *	1.0	10,509	2	21,018
Arginine	" †	1.8	9,676	2	19,352
Aspartic	" ‡	1.8	7,394	3	22,182
Hydroxyglutamic	" ‡	2.5	6,524	3	19,572
Valine	" *	1.9	6,165	3	18,495
Sulfur	" §	0.6	5,345	4	21,380
Tyrosine	" ‖	5.6	3,235	6	19,410
Phenylalanine	" ‡	7.6	2,173	9	19,557
Proline	" *	9.0	1,279	15	19,185
Alanine	" **	3.8			
Leucine	" ‡	25.0	525	37	19,425
Glutamic	" ‡	31.3	470	41	19,270
Ammonia	" *	3.6	473	41	19,393
Cystine	" ‖	0.5		?	

* Osborne and Liddle, quoted from Dakin (9).

† Kossel and Kutscher, quoted from Dakin (9).

‡ Dakin (9).

§ Osborne (4), p. 87.

‖ Folin and Looney (10).

** In view of Dr. Dakin's recent careful redetermination, Dr. Osborne doubts the accuracy of the higher figure, 9.8 per cent, obtained by Osborne and Liddle.

tent of casein in estimating its minimal molecular weight, and at that time explained in detail the method of calculation (1). In Table I we have used the analysis of zein published by Dakin (9) for the calculation of the minimal molecular weight of that protein. This we have supplemented with the colorimetric tyrosine and cystine

determinations of Folin and Looney (10). Cystine and histidine would seem to be the amino acids in which zein is poorest. If zein contains 0.8 per cent of histidine the molecular weight of zein cannot be less than 19,387. On the other hand, if newer analyses increase the amount of this amino acid the probable minimal molecular weight, as estimated from histidine, would of course become smaller.

There is some reason to believe that no very great change will occur in this estimate of the minimal molecular weight. For if the zein molecule contains 2 molecules of arginine its weight would be 19,344; 3 molecules of β-hydroxyglutamic acid bring the molecular weight to 19,752; 3 of aspartic acid to 22,182; and 4 atoms of sulfur to 21,380 (4).[3] Although it is probable that all of these values will change as methods of separation and analysis improve, the very high known percentage composition of zein on the one hand, and the frequency with which a small number of assumed molecules leads to a molecular weight near 20,000 allows us to assume a figure of this order for the minimal molecular weight of zein with a fair degree of probability.

The true molecular weight of zein may be very much larger than 20,000. Folin and Looney (10) have found only 0.5 per cent of cystine in zein hydrolysates by their colorimetric method. If this estimate is correct the molecular weight cannot be less than 48,040. Osborne (4,[3] 11) had previously found that zein contained but a small amount of sulfide sulfur, and had accordingly predicted a high molecular weight. He found but 0.212 per cent of sulfide sulfur. On the basis of this determination 15,127 gm. of zein would contain but 1 such atom. Three times this value leads to a molecular weight of 45,381, which is in good agreement with the molecular weight postulated on the basis of the cystine content. This coincidence lends a certain weight to both values, and also suggests that all of the sulfide sulfur in zein cannot represent cystine (4,[4] 12). In the case of zein, as in that of casein, it seems preferable to pro-

[3] Osborne (4), p. 87.
[4] Osborne (4), p. 88.

ceed on the basis of a minimal molecular weight, and await further investigation before speculating as to the true molecular weight of this protein.

III.

The Dicarboxylic Amino Acids in Zein.

On the basis of a minimal molecular weight of approximately 19,400 the number of molecules of each amino acid contained in zein has been estimated. The resultant molecular composition of the protein, recorded in Table I, is perhaps more easily interpretable than the percentage composition. Thus the analytical results indicate that on the basis of a molecular weight of 19,400 zein contains 3 molecules of aspartic acid, 3 of β-hydroxyglutamic acid, and 41 of glutamic acid; or in all 47 dicarboxylic acids. On the assumption that the 41 ammonia molecules represent amide-bound carboxyl groups, these must be subtracted from the 47 dicarboxylic acids to yield the number of free carboxyl groups derived from this source. The excess of dicarboxylic acids over ammonia leaves six such groups. There may, of course, be more free acid groups in zein. In making a similar estimate for casein we pointed out that it "is possible that further advances in the methods of extracting and separating the amino acids may increase even the high yields that Dakin has now obtained. It is conceivable that such amino acids as tyrosine and cystine may retain a certain capacity to combine base when held in polypeptide linkage. Finally certain monoamino monocarboxylic acids may be so oriented in the protein molecule as to yield terminal groups. On the other hand, certain of the dicarboxylic acids might exist in such firm combination with the terminal groups of diamino acids, that they would not bind base at the alkalinities that we have reached" (8).[5]

In a subsequent section measurements of the base-combining capacity of zein are reported and compared with what might be expected from the composition of the protein. This comparison involves no assumption regarding the minimal molecular weight of zein, for the free dicarboxylic acids can be directly calculated from

[5] Cohn and Berggren (8), p. 48.

the percentage composition. This has been done in Table II. Dividing the percentage of the amino acid in a protein, by the molecular weight of the amino acid yields as quotient the number of molecules of the amino acid in the protein. By adding together the molecules of the dicarboxylic amino acids and subtracting the molecules of ammonia we come to the conclusion that the free groups of dicarboxylic acids in 1 gm. of zein should combine approximately 30×10^{-5} mols of base.

IV.

The Characterization of Zein.

Two zein preparations from two different laboratories have been used in these experiments, and have given closely concordant re-

TABLE II.

Calculation of Free Acid Groups in Zein.

	Dicarboxylic amino acids in zein.				Amide-bound dicarboxylic acids.	Free dicarboxylic acids.
	Glutamic acid.	Aspartic acid.	Hydroxyglutamic acid.	Total.	Ammonia.	
	(*a*)	(*b*)	(*c*)	(*a*+*b*+*c*)	(*d*)	(*a*+*b*+*c*−*d*)
Amount found in zein, *per cent*....	31.3	1.8	2.5	35.6	3.6	
Mols in 1 gm. of zein, *mols* × 10^{-5}	212.8	13.5	15.3	241.6	211.5	30.1

sults. For one we are indebted to Dr. T. B. Osborne, and for the other to Dr. J. M. Looney. The former, with which most of the work has been done, was prepared by Mr. L. S. Nolan at the Connecticut Agricultural Experiment Station in 1923. Dr. Looney's zein was prepared in connection with the investigation of Folin and Looney already alluded to of "colorimetric methods for the separate determination of tyrosine, tryptophane, and cystine in proteins" (10).

The dry zein obtained from these different sources was transfered to salt mouth Nonsol bottles; wetted with distilled water, and mechanically stirred. Toluene was always added to prevent bacterial

action. When the zein had settled completely, the supernatant liquid was decanted, and the solid material ground with pestle and mortar until quite fine. The finely subdivided precipitate was then returned to the Nonsol bottle and washed with repeated changes of distilled water. The zein was brought into equilibrium with each successive wash water by prolonged high speed mechanical stirring. The wash waters were tested for chloride and sulfate, and the hydrogen potentials measured. The zein was not used until water

TABLE III.

No. of washings.	Osborne's zein.			Osborne's zein reprecipitated.			Looney's zein.		
	Chloride.	Sulfate.	pH+	Chloride.	Sulfate.	pH+	Chloride.	Sulfate.	pH+
1	0	0	5.8	+	0	5.7	+	0	
2			5.8	+		5.5	+		
3			5.7	+		5.5	+		4.4
4			5.7	+		5.8	+		
5			5.7	0		5.8	?		4.5
6							?		4.5
10							?		4.5
12									4.8
13									5.0
14									5.5
15									5.2
16							0		5.6
17									5.7

saturated with it had a constant hydrogen potential, and was free from chloride and sulfur. Osborne's zein was free from chloride and sulfur, and produced a hydrogen potential in water from pH 5.7 to 6.0. The different samples of this preparation that have been used were always washed from five to ten times. Looney's zein was free from sulfate, but at first contained a trace of chloride. The first seven wash waters of this preparation had a pH of 4.5. By the tenth washing the chloride had disappeared. The water saturated with the preparation became progressively more nearly neutral during the next five washings. The record of these changes is reported in Table III.

After the fifteenth washing the pH produced by this preparation was identical to that produced by Osborne's zein. Presumably

the two preparations had been brought to the same physical state in which they "dissolved in water to a constant and characteristic extent when the amount of protein precipitate with which the solution was in heterogeneous equilibrium was varied within wide limits" and in which "the dissociated ions of the dissolved protein give a hydrogen ion concentration to water" (13)[6] of approximately 2×10^{-6} N.

The solubility in water of Osborne's zein was determined in three different experiments. The amount of saturating body was varied

TABLE IV.

Solubility of Zein in Water.

Preparation and experiment No.	Date.	Zein nitrogen suspended in 100 cc.				
		mg. 13.8	mg. 27.6	mg. 34.5	mg. 57.5	mg. 69.0
		Zein nitrogen in 25 cc. of filtrate.				
	1923	mg.	mg.	mg.	mg.	mg.
O-1	June 7.	0.24	0.22			0.23
		0.21	0.22			0.24
		0.22	0.22			0.23
	1924					
O-2	Feb. 21.			0.20	0.22	
				0.20	0.22	
O-3	Mar. 24.			0.22	0.22	
				0.20	0.20	
				0.20	0.20	
	Average.....	0.22	0.22	0.20	0.21	0.23

from 1 to 5, without affecting the solubility of the protein. In Experiment 3 a fraction of zein was employed that had previously been dissolved in sodium hydroxide, reprecipitated with hydrochloric acid, and again washed with water. Although this treatment altered certain of the physical properties of the zein it had no measurable effect upon the solubility of zein in water.

The average of these experiments yields a value of 0.22 mg. of N in 25 cc. or 8.8 mg. of N in 1 liter for the solubility of zein in water

[6] Cohn (13), p. 721.

at 25°C. Since zein contains 16.17 per cent of nitrogen (4) only 0.054 gm. of zein dissolved in 1 liter of water; the smallest solubility of any protein, other than paracasein, that we have thus far detected.

V.

The Base-Combining Capacity of Zein.

Zein combines with sodium hydroxide and passes into solution. The strength of the acid groups in zein, and the equivalent combining weight of zein for base are being investigated by means of solubility and electromotive force measurements upon systems containing an excess of the protein. For the present we are concerned with the relation between the dicarboxylic amino acids in zein and its maximal base-combining capacity. Accordingly only those experiments are reported in which the alkali was in excess of the protein. Under these circumstances all the zein was dissolved.

The methods that have been employed in measuring and in calculating the base-combining capacity of zein from electromotive force measurements have already been described in detail in connection with a similar study upon casein. "If one knows the original concentration of the basic solution, and the amount of the protein it contains, the determination of the residual free base suffices for the calculation of the base bound by the protein. The free base in solution can be directly determined by electromotive force measurements of the hydrogen electrode against any known standard" (8).[7]

Measurements are reported in Table V of the electromotive force between 0.1 N calomel electrodes and hydrogen electrodes in sodium hydroxide solutions containing zein. From these measurements, and the temperatures at which they were made, the hydroxyl potentials have been calculated by means of the relation

$$pK_w - pOH^- = \frac{\text{E.M.F. observed} - \text{E.M.F. 0.1 calomel}}{0.001983\ T} \quad (1)$$

[7] Cohn and Berggren (8), p. 49.

For the negative logarithm of the dissociation constant of water, pK_w, the Sörensen-Michaelis (14)[8] values have been tentatively adopted; and for the 0.1 N calomel electrode the values calculated by Clark (15) from Lewis, Brighton, and Sebastian's study of the normal electrode. For the reasons that have led us to employ these estimates of the constants reference must be made to the earlier communication (8).

The free sodium hydroxide in systems containing zein, and an excess of alkali, have been estimated from the hydroxyl potential measurements, by means of the relation

$$\log \frac{1}{(NaOH)} = pNaOH = pOH^- - p\gamma \qquad (2)$$

in which pNaOH is the negative logarithm of the sodium hydroxide concentration, and $p\gamma$ the negative logarithm of the activity coefficient of the hydroxyl ion. Some doubt obtains as to the activity coefficients of the hydroxyl ions in solutions of mixed electrolytes, for reasons that have already been considered. For the present we have assumed that the activities were determined by the total concentration of base in the systems, and have adopted the γ values of the hydroxyl ion of Lewis and Randall (16).[9]

The difference between the free sodium hydroxide, and the total sodium hydroxide in these systems yields the base bound by the zein. The amount of base bound by the zein was very low in every experiment, so low as to increase appreciably the error involved in such determinations. This was smallest in the experiments in which 2 per cent of zein was employed, but all of the results with both preparations of zein were closely concordant. The average base-combining capacity of all the experiments with Looney's zein (designated by an L in Table V) was 0.0031, and of Osborne's (designated by an O) 0.0028 mols of sodium hydroxide per gm. The equivalent weights of zein bound by 1 mol of sodium hydroxide were therefore 3,226 and 3,571, respectively. The results of the most accurate experiments, in which the basic solutions contained most zein and least alkali, indicate that this divergence was attributable to the

[8] Michaelis (14), p. 23.

[9] Lewis and Randall (16), p. 382.

TABLE V.

Maximum Base-Combining Capacity of Zein.

Preparation and experiment No.	Zein in 1 liter.	Concentration of NaOH.	E.M.F.	Temperature.	pOH−	pNaOH (pOH− − pγ)	Concentration of free NaOH.	NaOH bound.	NaOH bound by 10 gm. of zein.
	gm.	N.	*volts*	°C.			N.	*mols*	*mols*
O-1	10.30	0.0410	1.0675	21.9	1.491	1.426	0.0375	0.0035	0.0034
			1.0687	22.0	1.471	1.406	0.0393	0.0017	(0.0017)
			1.0682	21.7	1.478	1.413	0.0386	0.0024	0.0023
			1.0681	21.5	1.479	1.414	0.0385	0.0025	0.0024
O-2	10.30	0.0205	1.0494	21.8	1.800	1.749	0.0178	0.0027	0.0026
			1.0492	21.6	1.802	1.751	0.0177	0.0028	0.0027
			1.0488	21.7	1.810	1.759	0.0174	0.0031	0.0030
O-3	20.78	0.0205	1.0446	21.4	1.879	1.828	0.0149	0.0056	0.0027
			1.0435	21.4	1.898	1.847	0.0142	0.0063	0.0030
			1.0437	21.4	1.895	1.844	0.0143	0.0062	0.0030
			1.0436	21.4	1.896	1.845	0.0143	0.0062	0.0030
O-4	24.40	0.0106	1.0127	21.9	2.428	2.392	0.0041	0.0066	0.0027
			1.0117	21.7	2.446	2.410	0.0039	0.0067	0.0028
			0.0074	21.4	2.516	2.480	0.0033	0.0073	0.0030
								Average..	0.0028
L-12	9.80	0.01	1.0279	20.2	2.160	2.124	0.0075	0.0025	0.0026
			1.0255	19.3	2.195	2.159	0.0069	0.0031	0.0032
			1.0253	19.6	2.201	2.165	0.0068	0.0032	0.0033
			1.0256	20.0	2.200	2.164	0.0069	0.0031	0.0032
L-13	10.42	0.02	1.0470	20.0	1.831	1.780	0.0166	0.0034	0.0033
			1.0465	19.4	1.835	1.784	0.0164	0.0036	0.0035
			1.0476	20.3	1.823	1.772	0.0169	0.0031	0.0030
L-10	21.20	0.02	1.0433	22.4	1.908	1.857	0.0139	0.0061	0.0029
			1.0429	21.4	1.908	1.857	0.0139	0.0061	0.0029
			1.0420	22.3	1.930	1.879	0.0132	0.0068	0.0032
								Average..	0.0031

large experimental error introduced by small differences in the electromotive force measurements, when the base-combining capacity was low. The average of experiments L-10 and L-12 was 0.0030,

and of experiments O-3 and O-4 was 0.0029 mol sodium hydroxide per gm. of zein. The corresponding equivalent combining weights become 3,333 and 3,448. The average of these, which is almost identical to that derived from the average of all the measurements, suggests that 3,400 may be taken as the equivalent combining weight of zein for sodium hydroxide. Six times this equivalent weight leads to the value 20,400 which is in good agreement with the minimal molecular weight that has been estimated from the amino acid composition of the protein. On the basis of a minimal molecular weight of this order these measurements of the base-combining capacity of zein therefore indicate that zein contains six acid groups. This number of groups exactly corresponds with the number of dicarboxylic acids that are not amide-bound, and that analyses have thus far revealed in zein hydrolysates.

This relation between the base-combining capacity of zein and its content of free groups of dicarboxylic acids is independent of any assumption regarding the minimal molecular weight of the protein. In Table II the concentration of free acid groups was calculated directly from the percentage composition of zein. The resultant concentration in 1 gm. of zein was found to be 30.1×10^{-5} N. The base-combining capacity of both of the zein preparations that have thus far been studied was of this order. As a first approximation it is therefore permissible to conclude that the base-combining capacity of this protein was precisely what should have been expected from its composition, and from the classical theory of the structure of proteins.

VI.

The Acid-Combining Capacity of Zein.

Zein is not amphoteric in its behavior. It does not combine with dilute acids, nor is it appreciably dissolved by them. Presumably the basic groups that zein contains are not free, at least at the acidities that have been reached in these experiments. Zein is thus possessed of a characteristic almost unique among proteins, of having no apparent basic properties.

The methods that have been employed in studying the acid-combining capacity of zein were very similar to those that were utilized in measuring its base-combining capacity. The zein was brought into equilibrium with different concentrations of hydrochloric acid in 100 cc. volumetric flasks by constant and prolonged agitation in

TABLE VI.

Acid-Combining Capacity of Zein.

Preparation and experiment No.	No. of washings.	Zein in 1 liter.	Concentration of HCl.	E.M.F.	Temperature	pH+	HCl	Concentration of free HCl.	HCl bound
		gm.	N.	*volts*	°C.			N.	*mols*
O-5	1	10.00	0.04	0.4211	22.0	1.458	1.405	0.0394	0.0006
				0.4214	22.0	1.463	1.410	0.0389	0.0011
	2			0.4196	21.8	1.433	1.380	0.0417	−0.0017
				0.4215	22.3	1.463	1.410	0.0389	0.0011
	3			0.4203	22.4	1.442	1.389	0.0408	−0.0008
				0.4208	22.8	1.450	1.397	0.0401	−0.0001
O-6	1	10.00	0.02	0.4390	23.0	1.759	1.713	0.0194	0.0006
				0.4372	22.5	1.732	1.686	0.0206	−0.0006
				0.4386	20.4	1.765	1.719	0.0191	0.0009
	2			0.4360	21.2	1.717	1.671	0.0213	−0.0013
				0.4377	21.4	1.745	1.699	0.0200	0.0000
	3			0.4378	21.9	1.744	1.698	0.0200	0.0000
				0.4379	22.6	1.743	1.697	0.0201	−0.0001
O-7	1	10.00	0.01	0.4564	22.7	2.058	2.022	0.0095	0.0005
				0.4557	22.5	2.047	2.011	0.0098	0.0002
	2			0.4550	21.7	2.039	2.003	0.0099	0.0001
				0.4547	21.3	2.037	2.001	0.0100	0.0000
	3			0.4551	21.7	2.040	2.004	0.0099	0.0001
				0.4556	22.2	2.046	2.010	0.0098	0.0002
								Average..	0.0000

a shaking machine. When it was believed that equilibrium had been established the zein was centrifuged to the bottom of the volumetric flasks, the supernatant liquid withdrawn for analysis, and replaced by hydrochloric acid of the original concentration. This hydrochloric acid was then saturated with the zein, the supernatant

liquid freed of precipitate and analyzed. In one set of experiments this operation was repeated three times.

Measurements of two kinds were usually made on the successive fractions of acid that were saturated with zein. The solubility of the zein was determined, and the amount of the acid that had combined with the protein estimated by means of electromotive force measurements. These are recorded in Table VI, which is arranged in precisely the same manner as Table V. These calculations in-

TABLE VII.

Solubility of Zein in Hydrochloric Acid.

Preparation and experiment No.	Date.	Concentration of hydrochloric acid: N.		
		0.01	0.02	0.04
		Zein nitrogen in 25 cc. of filtrate.		
	1924	*mg.*	*mg.*	*mg.*
O-5, 6, 7	Mar. 4.	(0.47)	0.30	0.32
		0.22	0.24	0.22
			0.27	0.27
O-5, 6, 7	" 7.	0.24	0.37	0.47
		0.31	0.31	0.35
		0.28	0.34	0.41
O-5, 6, 7	Apr. 11.	0.13	0.29	(0.46)
		0.17	0.21	0.29
		0.17	0.26	0.18
L-14, 15, 16	" 9.	0.22	0.18	0.18
			0.17	0.17

dicate that within the accuracy of the measurements, the zein bound none of the acid. The hydrogen potentials of the hydrochloric acid solutions were not measurably changed by the presence of the protein.

The solubility measurements also showed no perceptible effect of the hydrochloric acid (Table VII). The nitrogen determinations in this series were less concordant than are sometimes obtained, perhaps because of the granular nature of the zein precipitate, and the difficulty of bringing it into equilibrium with a solvent. Quadrupling the concentration of the acid did not appreciably affect the

solubility of the zein, however, nor did the number of times it was washed in acid solution. Finally, these solubilities were of the same order, though in certain cases slightly higher than the solubility of zein in water.

VII.

The Effect of Alkali upon Zein.

When, in the course of its preparation, casein is brought to an alkaline reaction, its base-combining capacity is increased by one-

TABLE VIII.

Maximum Base-Combining Capacity of Zein.

Preparation and experiment No.	Zein in 1 liter.	Concentration of NaOH.	E.M.F.	Temperature.	pOH^-	$pNaOH$ ($pOH^- - p\gamma$)	Concentration of free NaOH.	NaOH bound.	NaOH bound by 10 gm. of zein.
	gm.	N.	*volts*	°C.			N.	*mols*	*mols*
O-8	12.34	0.010	1.0220	22.0	2.269	2.233	0.0059	0.0042	0.0034
			1.0223	22.3	2.266	2.230	0.0059	0.0041	0.0033
			1.0200	22.7	2.306	2.270	0.0054	0.0046	0.0037
			1.0218	22.5	2.274	2.238	0.0058	0.0042	0.0034
O-9	14.26	0.020	1.0450	21.9	1.876	1.825	0.0150	0.0050	0.0035
			1.0460	21.8	1.858	1.807	0.0156	0.0044	0.0031
			1.0459	22.6	1.863	1.812	0.0154	0.0046	0.0032
			1.0456	22.2	1.867	1.816	0.0153	0.0047	0.0033
								Average..	0.0034

fourth (8), presumably because of the hydrolytic opening of bound groups. Zein is much poorer both in free dicarboxylic and in diamino acids than is casein. Provided the internally bound groups in casein represented cyclic anhydrides, one would expect alkali to have a lesser effect upon the base-combining capacity of zein. On the other hand, as experiments upon solubility and acid-combining capacity indicate, zein behaves as though it possessed no free amino groups, while analyses reveal the presence of both arginine and histidine. The former is present to the extent of 1.8 per cent and the latter to 0.8 per cent. Each gram of zein must, therefore, contain 5.2×10^{-5} mols of histidine, and 10.3×10^{-5} mols of arginine. Even if

Bracewell (17) is correct in stating that the histidine in the protein molecule does not combine with acid, the arginine in zein, were it free, should bind 10.3×10^{-5} mols of acid, and together they could combine 15.5×10^{-5} mols of acid. The acid-combining capacity of zein could therefore be one-third, or even one-half, as great as its base-combining capacity.

In order to test the effect of reaction upon base-combining capacity of zein a sample of Osborne's zein was dissolved in sodium hydroxide. Zein is a very weak acid, so weak as not to be readily soluble in ammonium hydroxide, and did not dissolve in sodium hydroxide until the hydrogen potential was greater than 10. The dissolved zein was therefore brought to a much more alkaline reaction than is needed to open the groups bound in native casein, was filtered, again reprecipitated, and washed free from the accumulated salt. The appearance of this preparation was appreciably different from the original material. When it was dissolved in sodium hydroxide solutions, however, it combined with very little, if any more base, than saturated zein. The results of two experiments made with this preparation are reported in Table VIII. The average of these results is a little higher than the average of the results in Table V. The order of the difference is, however, not greater than the experimental error, and it is only possible to conclude from these experiments, therefore, that if untreated zein combines six basic equivalents, zein treated with alkali binds six or seven groups. On the basis of a molecular weight of 19,400 zein possesses at most one, but more probably no, internally bound group that is readily liberated by alkali, whereas it contains at least 2 arginine and 1 histidine molecules.

The relation of zein to acids also seems to have been unaltered by this alkaline treatment. Experiments upon the solubility, and upon the acid-combining capacity of this fraction are recorded in Tables IX and X. Zein so treated did not combine acid, nor did it dissolve in acid more than in water. All of the amino groups of the diamino acids must therefore be firmly bound in the zein molecule, more firmly at least than are certain of the groups in casein.

If these groups are bound by acid groups, either zein contains a slightly greater concentration of dicarboxylic acids than have thus

far been detected, or the cystine or tyrosine, or possibly certain internal groups, are here functioning as acids. It might be added that recent investigators (18) regard tyrosine as a divalent acid, and that there is enough of this amino acid in zein to account for the apparent discrepancy. Although one might speculate further

TABLE IX.

Solubility of Zein in Hydrochloric Acid.

Preparation and experiment No.	Date.	Concentration of hydrochloric acid: N.			
		0.0	0.01	0.02	0.04
		Zein nitrogen in 25 cc. of filtrate.			
	1924	*mg.*	*mg.*	*mg.*	*mg.*
O-3	Mar. 24.	0.22	0.20	0.21	0.20
		0.20	0.20	0.19	0.20
		0.20	0.20	0.20	0.20

TABLE X.

Acid-Combining Capacity of Zein.

Preparation and experiment No.	Zein in 1 liter.	Concentration of HCl.	E.M.F.	Temperature.	pH^+	pHCl	Concentration of free HCl.	HCl bound.
	gm.	N.	*volts*	*°C.*			N.	*mols*
L-14	2.08	0.01	0.4562	21.8	2.059	2.026	0.0095	0.0005
			0.4559	23.3	2.044	2.008	0.0098	0.0002
L-15	2.08	0.02	0.4373	22.8	1.731	1.685	0.0207	−0.0007
			0.4378	23.2	1.738	1.692	0.0203	−0.0003
L-16	2.08	0.04	0.4202	22.6	1.440	1.387	0.0410	−0.0010
			0.4204	22.8	1.443	1.390	0.0407	−0.0007
							Average..	−0.0003

regarding the method by which these bases are combined in the protein molecule, it seems preferable to await those analytical advances, which must, of necessity, yield the final answer to this problem.

It is thus apparent that up to the present it has not been possible completely to correlate behavior and composition. Our investigations indicate, however, that zein, a protein with a very low concentration of diamino acids, has no apparent basic properties; and that its acid properties are such as might be expected from the dicarboxylic acids that it contains that are not bound as amides. Finally the extremely small concentration both of acid and of basic groups, and the very low solubility of zein in water, and in dilute solutions of acids and alkalies, characterize zein as a very inert protein, more inert than any other that we have thus far investigated.

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THE INFLUENCE OF ELECTROLYTES ON THE STABILITY OF RED BLOOD CORPUSCLE SUSPENSIONS.*

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(Accepted for publication, August 6, 1924.)

The experiments which are described in this paper are concerned with the electric charges of red blood corpuscles in fluid suspensions. They are based on the work of those investigators who have determined the physical properties of suspensions and colloidal solutions and are an attempt to apply these general principles to the special case in hand.

It was Jevons (1) who first suggested electrical repulsions as a source of suspension stability and Hardy (2) who pointed out the relation of the isoelectric point to flocculation, while the quantitative measurements of Powis (3) and Ellis (4) demonstrated that a critical potential exists for suspended oil droplets below which a suspension of them is no longer stable.

An application of these principles to biological phenomena has been recently made by Northrop and De Kruif (5), who have shown that a similar critical potential exists, under certain conditions at least, for suspensions of bacteria. In the case of suspensions of red blood cells, however, the application of these basic principles has been less successful. The possibility of such a physical explanation for the suspension stability of the blood has aroused the attention of clinicians recently and Fåhraeus (6), to whom the revival of interest in this subject is due, at first suggested that the stability of the different bloods depended on differences in the electric charge of the cells. In his later studies he was unable, however, definitely to establish such a relationship. The agglutination of red cells by chemicals has also been assumed to be due to differences in electric charge, but here again Rona and György (7) were unable to demonstrate such a correlation in the case of ricin agglutination.

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* A preliminary report of this work appeared in *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 529.

In our previous studies of the agglutination of red cells by arsphenamine (8), it was suggested that the mechanism of the action might be due to variations in the electric charges of the cells. From this specific problem we have proceeded to that of the mechanism of agglutination by chemical agents in general. In the present paper we will demonstrate the variations that different salts produce in the charges of cells in suspension and discuss the correlation of these differences with the stability of the suspension.

Method.

The Cell Suspension.—The primary requisite for a study of the properties of a suspension of red cells is that they be suspended in a fluid which is isotonic with them, and which at the same time does not contain substances affecting the electric properties of the cells. Electrolytes and proteins must therefore be excluded. The nearest approach to this ideal condition is obtained by a repeated washing of the cells in isotonic sugar solution and a suspension of them in it.

Such a suspension is unstable. In the course of a few minutes the clumps resulting from the aggregation of the individual cells may be seen with the naked eye and these clumps rapidly settle to the bottom of the container. A preliminary washing of the freshly drawn blood with isotonic NaCl solution makes the occurrence of this phenomenon more certain, provided the salt is thoroughly removed by subsequent washings with sugar solution, since the presence of a small amount of this electrolyte will stabilize the suspension. The instability of the final suspension, therefore, becomes a valuable criterion of its suitability in the experiments to be described. If this is lacking, it is probable that the stability is due to the presence of some electrolyte and this renders a proper estimation of the effect of the experimental procedure impossible.

In our experiments rabbit blood was used. It was defibrinated and washed three times with 0.9 per cent NaCl solution, five times with 10 per cent sucrose solution, and made up to a 5 per cent suspension in this fluid. If the suspension remained stable and did not agglutinate in a few minutes, it was rewashed in the sugar solution or discarded.

The Measurement of Instability.

The quantitative expression of the degree of agglutination, that is, of the instability of the suspension, was the most difficult part of our experiments. After trials of various methods it was decided that reliable comparative values would be obtained if the degree of agglutination was expressed by three arbitrary symbols, +, ±, and 0. The symbol + means that agglutination was complete, the suspension being entirely unstable and the cells settling completely so as to leave a clear supernatant fluid. By ± is meant a degree of instability such that agglutination had occurred with definite settling, but in which the supernatant fluid still contained cells in suspension. When no gross agglutination and no settling greater than that observed in the most stable controls occurred, the value 0 was used. Any attempt at further subdivision was found to lead to uncertain results.

In our experiments the settling of the suspension is taken as a measure of its stability. Settling of the cells was observed, however, in the most stable cell suspensions, so that there is no qualitative distinction between the "normal" and the more rapid settling due to agglutination. The measurement of the agglutination, therefore, depends on the measurement of the velocity of the reaction, and if this is to be estimated by a single determination at one point in the process (for the purposes of comparison with the same process under different conditions) a point must be selected which will properly represent the differences in the rates.[1] If the observations are made too early or too late in the course of the reactions, significant differences will be missed. After considerable experimentation we decided that the degree of instability should be determined at a period of from ½ to 1 hour after the mixtures had been prepared. After this time settling has occurred to some extent in all tubes and the instability due to the experimental procedure is masked by the "normal" process of settling. Unless otherwise stated the tubes were placed in a water bath at 37°C. In certain experiments the tubes were kept at room temperature and observed at earlier intervals.

[1] *Cf.* Freundlich (14), p. 570.

The Electric Charge.

The degree of the electric charge of the cells was determined by cataphoresis. In our experiments the microscopic method was used as described by Ellis (4), Powis (3), and Michaelis (9). A cell 2.0 × 1.0 × 75.0 mm. was placed on the stage of a microscope in the ocular of which a micrometer had been inserted. Each extremity of the trough was connected by an agar siphon to a $Zn\text{-}Zn_2SO_4$ electrode. The cell was filled with the red cell suspension, covered with a glass slide, and a direct current of 115 volts applied. Measurements of the speed of the particles at the proper depths were made and averaged in the usual manner and the results expressed as millivolts by means of the usual formula.

The Experiments.

The procedure in all the experiments was the same. A 5.0 per cent suspension of red cells in isotonic sucrose was mixed with the same amount of solution containing decreasing concentrations of the various electrolytes to be studied. The series of tubes was allowed to stand at 37°C. for 1 hour. The cells were now resuspended in the proper sugar-electrolyte mixture and the degree of charge determined by cataphoresis.

Some preliminary experiments were performed in the same manner with collodion particles. These were a repetition of Loeb's experiments (10). The results are reported in order that a comparison of the properties of these particles may be made with those of a red cell suspension under the same conditions. The presence of the sugar in the suspension fluid made no apparent difference in either the stability of the suspension or in the electric charge of its particles. The pH value of the original suspension of particles before the addition of electrolyte was 5.0.

The Effect of Electrolytes on the Charge of Collodion Particles and Its Relation to the Stability of the Suspension.

The results of the experiments with collodion particles are shown in Fig. 1. It is seen that all substances depress the charge of the particles and that one of them, ferric chloride, reverses the charge. When

the P.D. is greater than a certain value, 32 mv., the suspension is stable; when lower than this critical value, aggregation of the particles

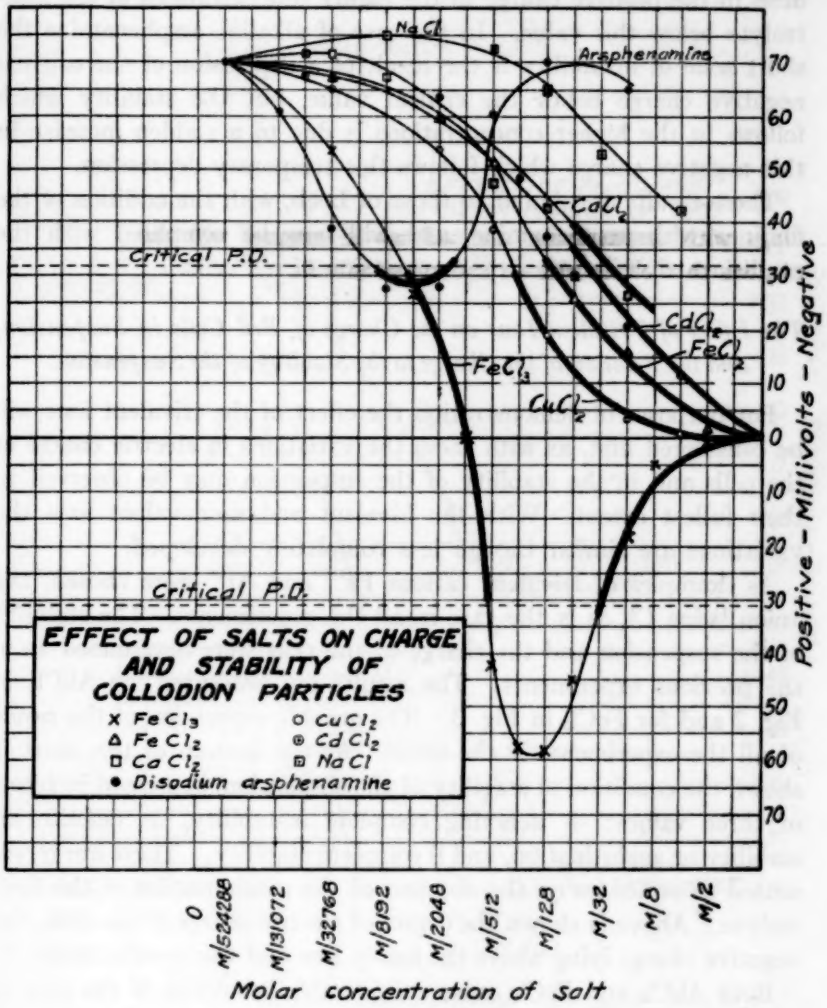

FIG. 1.

occurs and instability results. Conditions of both stability and instability alternate in the series of ferric chloride and arsphenamine. With ferric chloride this "irregular series" is produced by a secondary

rise in the reversed positive charge to a value above that of the critical value, and the following zone of instability is the result of a drop in the positive charge in the higher concentrations of the electrolyte below this value. In the case of alkaline arsphenamine the short zone of instability is the result of a depression of the original negative charge below the critical value, but the stability which follows in the higher concentrations is due to a sudden increase in this negative charge which follows this temporary depression.

These results are similar to those of Loeb, with the addition of the findings with arsphenamine, and will now be compared with the conditions that obtain with a suspension of red cells.

The Effect of Trivalent Ions on the Charge of Red Cells in Suspension, and the Relation of the Charge to the Stability of the Suspension.

For purposes of demonstration the effect of the trivalent ions will be considered first, as with them the variations in electric charge of the cells and in the stability of the suspension may be observed to their fullest extent. With the bivalent and monovalent ions the variations are similar though less completely developed.

As examples of trivalent cations Fe''' and Al''' were chosen, the anion being Cl, as is the case in all the experiments. The stability of the suspension and the charge of the cells were determined as in the previous experiments. The results are presented for Al_2Cl_6 in Fig. 2 and for $FeCl_3$ in Fig. 3. The graphic expression of the result of all the experiments is the same. At the bottom of the chart is shown the condition of stability of the suspension, expressed in terms of three values: + denoting complete instability, ± definite instability or agglutination, and 0 complete stability. These are represented as ordinates on the abscissæ of the concentration of the electrolyte. Above is shown the degree of electric charge of the cells, the negative charge lying above the heavy line and the positive below it.

Both Al_2Cl_6 and $FeCl_3$ cause considerable hemolysis of the cells in the high concentrations and this obscures the determinations of both stability and potential. A wider range of concentration is observed with Al_2Cl_6 but the type of variation is the same with both salts. There is a depression of the original negative charge which reaches 0 at

about M/32,000, followed by an increasing positive charge, which is in turn again depressed at a concentration of M/512. This secondary depression of the positive charge is less well shown by $FeCl_3$ because

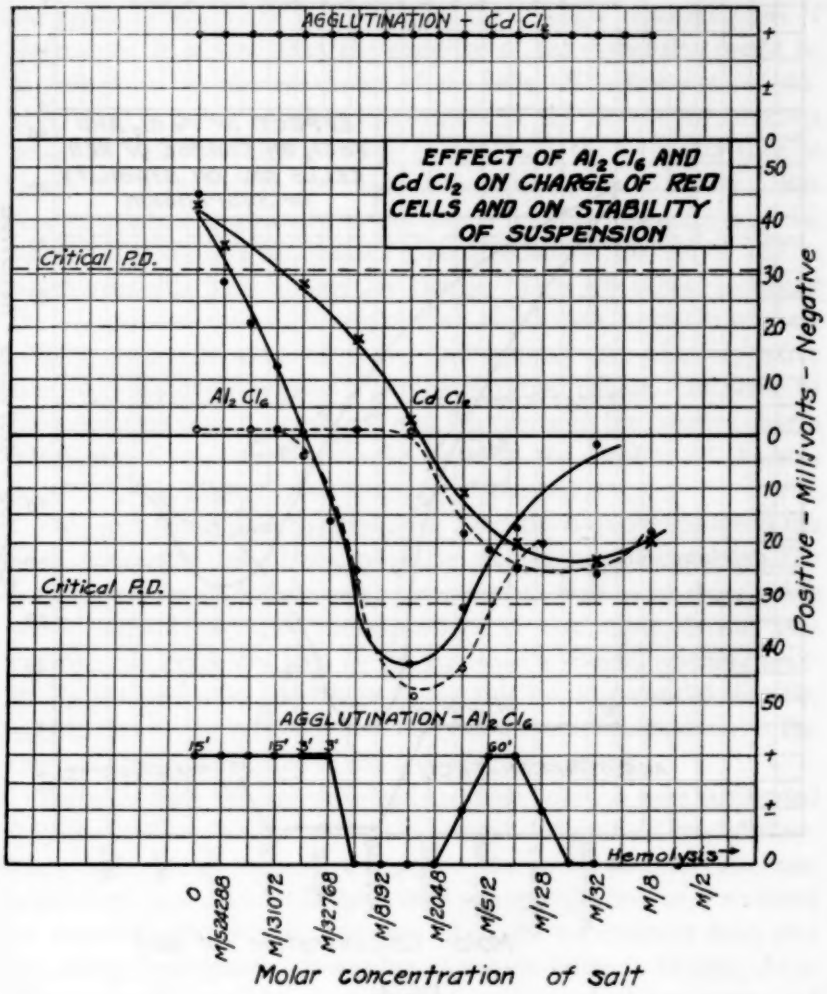

FIG. 2.

hemolysis obscures the readings in those tubes in which the changes would be most marked, although the beginning of the depression is readily seen.

The stability of the suspensions in the two salt solutions is also similar. In both, at a certain concentration of electrolyte the un-

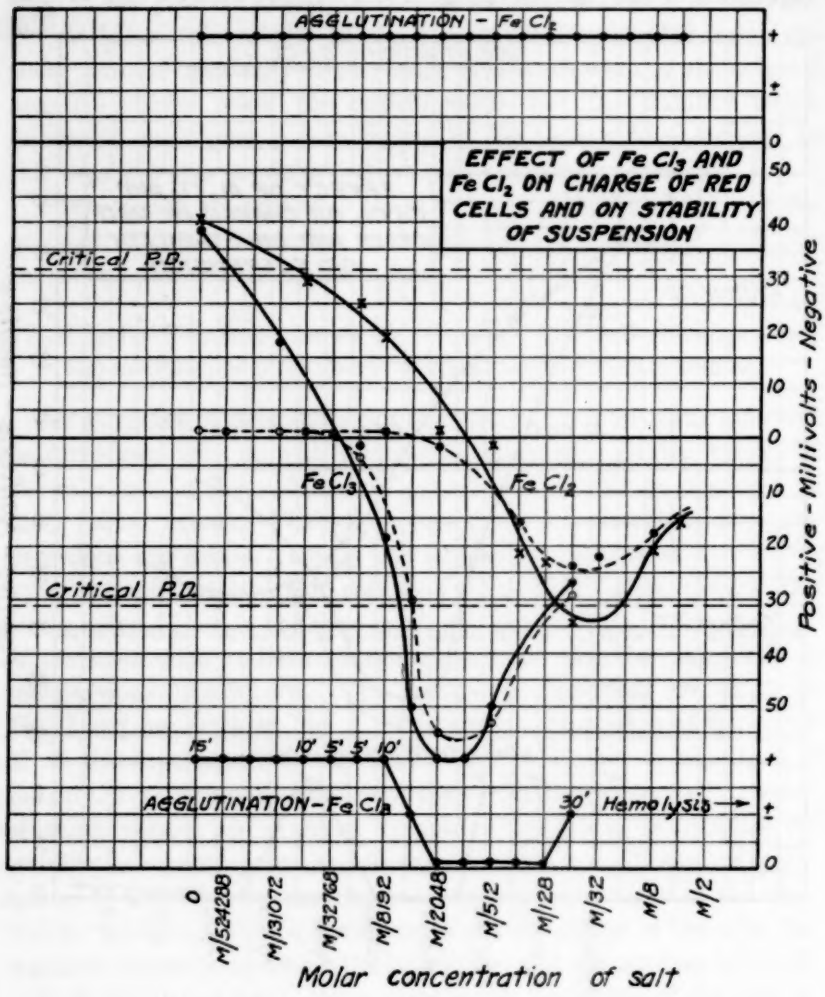

FIG. 3.

stable sucrose suspension becomes stable, and this is followed, in the higher concentrations, by a zone of instability. In the highest concentrations of the electrolytes, in which the cells are coagulated by

the action of the metallic salts and hemolysis is prevented, determinations are again possible, and stability occurs.

A comparison of the two phenomena, electric charge and stability of the suspension, gives a rather surprising result. In that half of the series in which the tubes contain the stronger concentrations of electrolyte, it is seen that the stability of the suspension might be explained by the electric charge of the cells. The first zone of stability that is noted as the concentration of the electrolyte increases coincides with the high positive charge, and the following zone of instability accompanies the depression of this positive charge. But this correlation apparently is not found in that half of the series whose tubes contain the weaker concentrations of electrolyte and in which the cells have a negative charge. Here all tubes, including the sucrose control which contains no electrolyte, agglutinate completely, although some of them, and notably the electrolyte-free control, apparently possess a strong negative charge. In some of the tubes this negative charge is equal in degree to the positive charge which may be assumed to be the cause of the stability of the suspension in the higher concentrations of the series.

This apparent paradox is explained when the negative charge in the weak concentrations of electrolyte is studied more carefully. The negative charge in the sucrose suspension, as well as in those tubes which contain only low concentrations of electrolyte, is not permanent. It gradually falls as the suspension stands, reaching practically the null point; and it is during this fall in potential that the aggregation of the cells and instability of the suspension occur. The changes are demonstrated in the following experiment.

The cells from the last washing in sucrose solution were suspended in this fluid, agitated, and their charge determined immediately. They were found to have a negative charge of 40 mv. The suspension was now allowed to stand for 5 minutes, the cells were removed by centrifugalization and resuspended in the supernatant fluid, and the charge determined. It was found to have fallen to 19 mv. After 10 minutes agglutination was observed in the original suspension and the determination was repeated and the charge found to be almost entirely absent, as more than 100 seconds were required for the cells to travel the required distance. These same cells were now sus-

pended in fresh isotonic sucrose solution and the charge was found to have risen to the original figure of 41 mv.

The same fall in charge occurred in the weaker concentrations of electrolytes, as shown by the following experiment. A series of tubes with increasing concentrations of Al_2Cl_6 and $FeCl_3$ was prepared as in the experiments just described and allowed to stand for 1 hour. The tubes were then centrifuged and the clear supernatant fluid removed. The cells were now resuspended in the supernatant fluid and also in fresh mixtures of similar electrolyte concentration and the charges determined immediately in both. The results are shown in Figs. 2 and 3. The charge in the old supernatant fluid is represented by the white circles and the dotted lines. The immediate charge of the same cells suspended in the same electrolyte-sucrose concentration is that previously recorded.

It will be noted in the weak concentrations of electrolyte up to the point at which reversal occurs, that the negative charge has fallen to 0, while there has been no change in the positive charges in the stronger concentrations. A comparison of these late curves of P.D. of the cells with the condition of stability of the suspension shows an essential agreement, except in the highest concentrations of electrolyte. Wherever there is a P.D. greater than 32 mv. stability of the suspension occurs, while with a lesser potential, aggregation and instability result. The critical potential observed by Powis and by Ellis for suspensions of oil droplets, by Loeb for collodion particles, and by Northrop and De Kruif for bacteria, holds for suspensions of red cells, except in high concentrations of electrolyte. There is this difference with a red cell suspension, however, that the initial charge in the weak concentrations of electrolyte is not permanent and that it is the final P.D. of the cells that is correlated with the stability or instability of the suspension.

The Effects of Hydrolyzing Salts with Bivalent Ions.

Two types of salts with bivalent ions were studied, those which in solution become acid in the stronger concentrations and those which remain neutral. Since the effect of the P.D. of the cells and the resulting changes in the stability of the suspensions are quite different with the two types of solutions they will be described separately.

The hydrolyzing salts with bivalent ions may be arranged in a series in which the degree of acidity decreases. In describing their

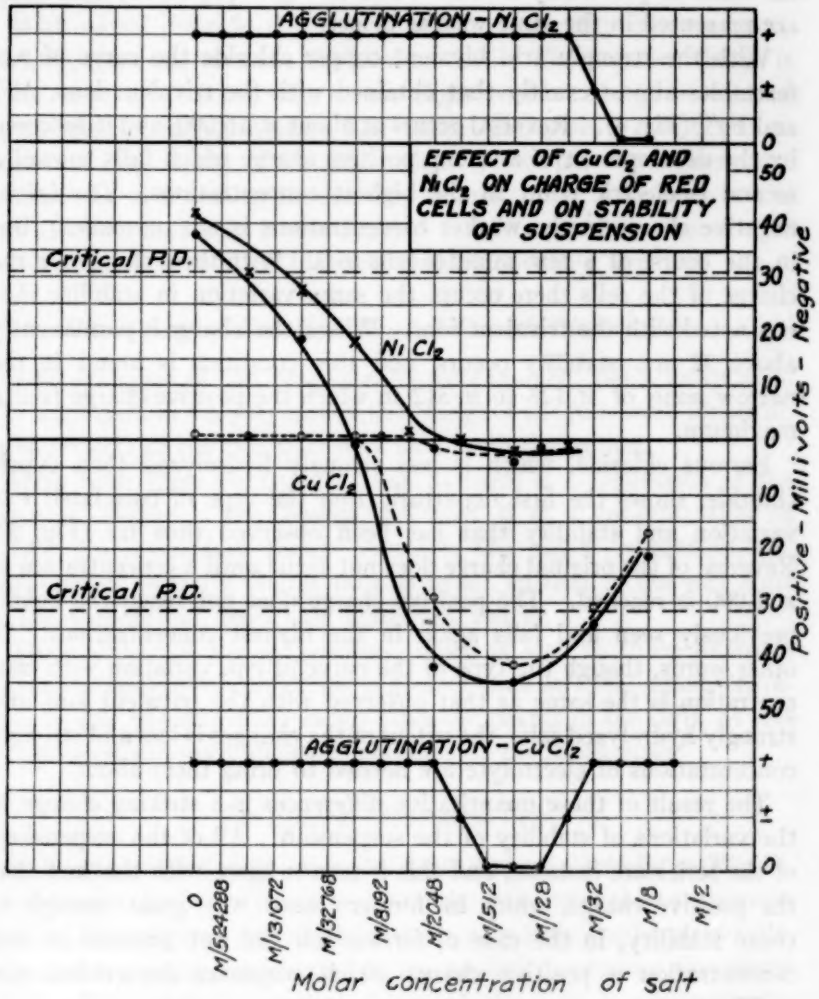

FIG. 4.

action we have considered them in this order, for in that way there is a gradual transition to the salts with bivalent ions which do not hydrolyze. The salts that were studied show the following order of

decreasing acidity in solution: copper chloride, ferrous chloride, cadmium chloride, nickel chloride. The experimental procedure was the same as previously described and in Figs. 2, 3, and 4, the results are presented in the same way as before.

With the strongly acid bivalent copper chloride the curve of P.D. resembles almost exactly that obtained with the trivalent ions, Al''' and Fe''' (Fig. 4). Reversal occurs at about M/16,000, and is followed by the development of a strong positive charge which falls toward a second isoelectric point in the highest concentrations. The initial negative charge in the weaker concentrations is not permanent, but in the course of a few minutes falls to 0. With these changes in the charge of the cells there occurs the same variation in stability that was noted with the trivalent ions. Where the charge is permanently above 32 mv. stability occurs, and this condition is found in the narrow zone of M/128 to M/512 in which the positive charge is at a maximum.

Ferrous chloride, which is less strongly hydrolyzed than cupric chloride, shows the first departure from the type of correlated P.D. variation and stability that has been observed thus far (Fig. 3). Reversal of the original charge does not occur until a concentration of M/1,000 is reached. The positive charge does not reach the height previously seen and falls again in the highest concentrations. In other words, though the type of the curve of P.D. variation with concentration is the same as that observed with the trivalent ions and strongly hydrolyzed salts, the extent of the changes is less and stronger concentrations of electrolyte are needed to bring them about.

The result of these quantitative differences is a striking change in the variations of stability of the suspension. All of the suspensions of the series are instable, and this is seen to agree with the fact that the positive charge, which in former cases was great enough to cause stability, in the case of ferrous ion did not produce at any concentration a positive charge which surpasses the critical P.D. of 32 mv.

Cadmium chloride (Fig. 2), which hydrolyzes less than ferrous chloride, shows an even greater departure from the fully developed variation in charge. Reversal occurs at M/1,000 and the positive charge is not great. The negative charge in the weak concentrations

is not permanent, so that in no suspension is the final charge greater than 32 mv. and as a result none of the suspensions are stable and all agglutinate.

With nickel chloride (Fig. 4), in which the acidity increases only slightly, the variations are the least marked. The negative charge is reversed at M/1,000 but only a very slight positive charge is acquired in the higher concentrations. The charge, therefore, is insufficient to cause stability in any of the suspensions, yet in the two highest concentrations, M/8 and M/16, stability occurs. It will be seen in the next section that this is the type of stability which is found with non-hydrolyzing salts of bivalent and monovalent ions.

The Effects of Non-Hydrolyzed Salts with Bivalent Ions.

Two examples of this type of salts were studied, $BaCl_2$ and $CaCl_2$. The variations in stability and in the charge of the cells were identical, so the effects of the two salts may be described together.

With both these electrolytes (Fig. 5) there is a depression of the negative charge. The charge becomes 0 at a concentration of M/64. No reversal of charge occurs in the higher concentrations. The original negative charge falls as usual, so that in the end none of the suspensions have any significant charge.

The stability of the suspensions, which is seen in those mixtures whose electrolyte concentration is greater than M/1,000, must, therefore, be due to some other factor than the charge of the cells, as this is depressed and even abolished throughout the stable zone.

The Effect of Monovalent Ions.

NaCl was studied as an example of this class of electrolytes (Fig. 5). Its action on the P.D. of the cells and on the stability of their suspension is of the same type as described for the non-hydrolyzing bivalent salts, except that greater concentrations are needed to produce the effects noted. The original negative charge of the cells is gradually depressed, reaching the isoelectric point at about M/8. The negative charge in the weaker suspensions falls, so that ultimately the cells in all the tubes are without charge. The stability is practically identical with that observed with Ba and Ca, being present in all suspensions in

which the concentration is greater than M/512, and it therefore bears no relation to the charge of the cells.

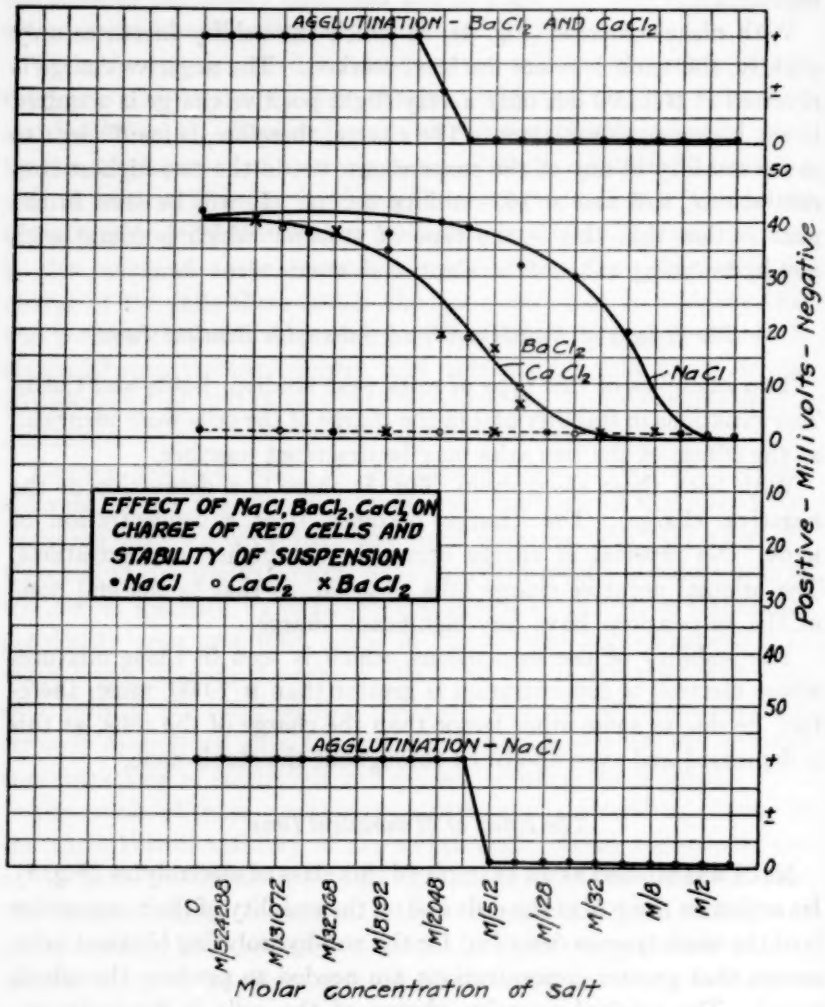

FIG. 5.

The Effect of Disodium Arsphenamine.

As stated in the introduction, our interest in the action of ions on the stability of red cell suspensions arose from a previous study (8)

of the agglutinating action of arsphenamine. We have therefore studied the action of this agent on the charge of the cells, though it differs considerably from the highly ionized salts whose effects have been described. This is particularly true of its chemical reaction, for its solutions are strongly alkaline.

The experimental procedure was the same as with the salt solutions. Fig. 6 shows the results that were obtained.

The immediately determined P.D. shows a gradual depression of the original negative charge which reaches its lowest point at about M/16,000. In contrast to the effects of all the salts previously studied the fall in potential does not continue to 0, but suddenly turns at M/16,000 and rapidly climbs to the highest value thus far observed. The late determination of charges shows that the negative charges developed in the weaker concentrations of electrolyte have all fallen to 0 as in the former experiments, but that the strong negative charge in the higher concentrations has persisted.

In its distribution with respect to concentration of disodium arsphenamine the zone of stability resembles that seen with the non-hydrolyzing bivalent and the monovalent salts. The original instability of the suspension persists until a concentration of about M/1,000 is reached, and from then on the suspension is stable. However, in spite of this similarity in the distribution of the stability the mechanism of its production is entirely different. Stability in this instance resembles that seen with trivalent ions and hydrolyzed salts, in that it depends on the charge of the cells. The stable zone coincides with the high negative charge produced by the alkaline arsphenamine, instead of with a strong positive charge as was the case with these salts. The instability in the weaker concentrations is due to the fall of P.D. to 0, a phenomenon exhibited by all suspensions irrespective of the electrolyte present. The stabilizing effect of arsphenamine in this experiment is very evident, but the question may well be raised if it can be considered the direct cause of the instability noted in any of the tubes, or in other words if it "agglutinates" the red cells *per se*. A careful examination of the tubes in which the suspensions are instable shows that in certain of them the instability is the direct result of the arsphenamine and not the result of the general loss of negative charge that is observed in weak concentrations of all electrolytes.

This was definitely demonstrated when the tubes were allowed to stand at room temperature instead of at 37°C. in the water bath, so

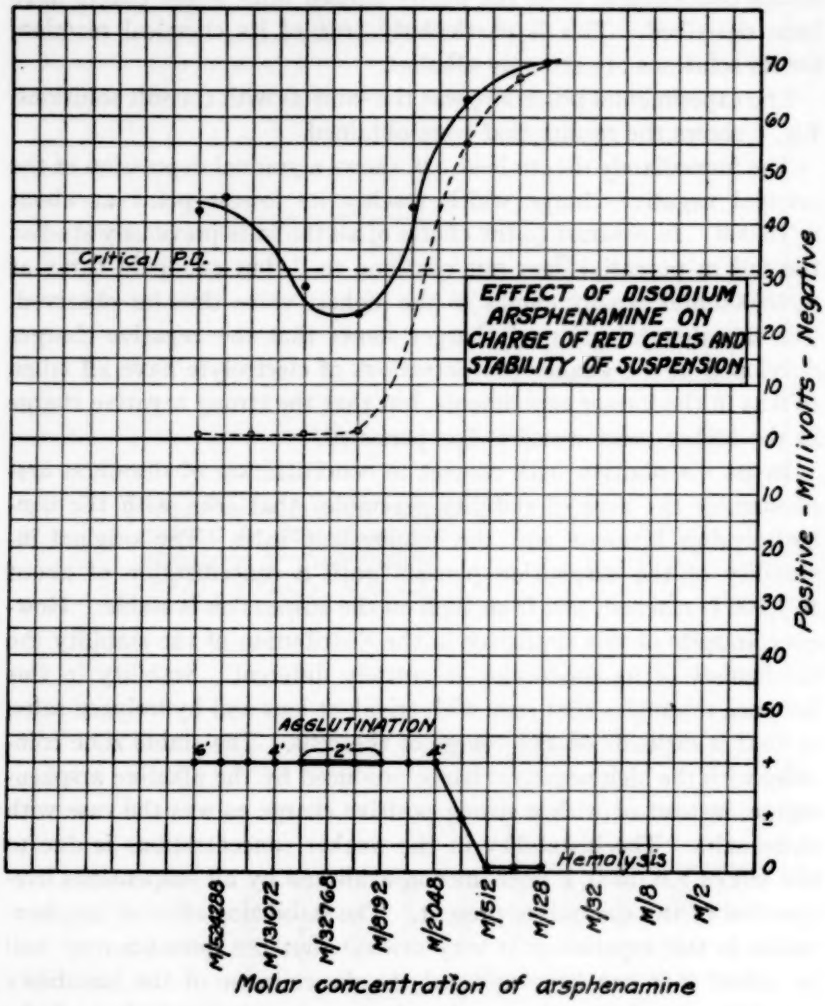

FIG. 6.

that agglutination of the cells and the resulting instability developed more slowly. Under these conditions it was found that in those tubes in which the original negative charge of the cells had been de-

pressed by the arsphenamine (M/32,000 to M/8,000) aggregation occurred considerably more rapidly (2 minutes) than in those in which the negative charge had not been so depressed (6 minutes). This increase in rate of agglutination is due to the immediate depression below the critical P.D. which occurs in these concentrations, whereas the agglutination which develops later is the result of the non-specific fall in negative charges that is found in weak concentrations of all electrolytes.

The occurrence of a rapid agglutination in certain of the suspensions in the instable zone was also found in the series of experiments with trivalent ions when the experiments were repeated at room temperature, the rate of the agglutination under these conditions not being so rapid as to obscure small differences. In Figs. 2 and 3 the time of appearance of aggregation of the cells is shown above the records of agglutinations. It is seen that in the neighborhood of the isoelectric point, agglutination occurred more rapidly than in those suspensions possessed of some charge. Agglutination of these was apparently delayed until the usual fall of P.D., which occurs in all suspensions weak in electrolyte, had brought them below the critical P.D. of 32 mv. With the hydrolyzing bivalent salts the differences were not well marked, probably due to the fact that the differences in charge are not so extreme as with the strongly active trivalent ions.

DISCUSSION.

The negative charge of red blood cells suspended in an isotonic solution of non-electrolyte, such as sucrose, is temporary, falling in the course of a few minutes to a point so low that it can no longer be accurately determined by the methods we have used. With this fall in the P.D. of the cells occurs aggregation, or agglutination, and the suspension becomes unstable. This impermanency of the negative charge is in striking contrast to the permanency of the negative charge of collodion particles when they are suspended in water, for there is no change in the P.D. of these particles and the electrolyte-free suspension is correspondingly stable for long periods. This difference presumably depends on differences in the mechanism of the production of the charge in the two cases and will be discussed at another time. For the present the fact alone will be considered. In this

discussion the charge which exists when the cells are first placed in the suspension fluid will be called the "immediate charge," and that which ultimately results when equilibrium is established will be termed the "final charge."

Electrolytes affect the charge of suspended red blood cells in the same manner as they affect that of all suspended particles. At certain concentrations they depress the charge to an isoelectric point. The negatively charged cells are apparently affected by the positive cation, for the valency of the latter determines in part the concentration of the salt which is required to bring about this result. Considerable variation was noted in each valency group as to the exact concentration of salt required. With trivalent salts M/32,000 to M/64,000 concentrations sufficed, while with the monovalent M/4 was required. Among the bivalent group wide variations occurred, from M/16,000 in the case of $CuCl_2$ to M/256 for $CaCl_2$, $FeCl_2$, and $CdCl_2$; $NiCl_2$ required M/1,000. Obviously some other factor than valency must enter into the cause of these wide variations, and this factor must operate only when the suspended particles are red blood cells, for no such discrepancies were noted in our experiments with collodion particles. Collodion particles were also much less susceptible than red cells to the action of the salts. For example, a concentration of M/1,000 $FeCl_3$ was necessary to bring them to the isoelectric point, whereas only M/32,000 was required in the case of red blood cells.

A possible explanation of these differences may be sought in the chemical composition of the two types of particles. The collodion particles are relatively inert, while the red cells, comprising both proteins and lipoids, act as amphoteric electrolytes and are therefore peculiarly subject to the effects of H ion concentration. Many of the salts whose actions were studied in our experiments hydrolyze, and in the higher concentrations their solutions are strongly acid. From the work of Loeb (11) and of Coulter (12) it is known that the combination of ions with gelatin and with red cells varies with the pH of the medium, not only quantitatively but also qualitatively. The addition of a salt which by hydrolysis increases the H ion concentration of the medium in which the cells are suspended will therefore modify the action of the ions present. It is our purpose to report later on the relative effects of H ions and of other cations and on the P.D. of the cells.

With certain electrolytes, notably those yielding trivalent ions, reversal of the charge occurs; a strongly positive potential is established, which falls again towards a second isoelectric point in the higher concentrations. The positive charge thus produced is a permanent one, and so differs from the negative charge in the weaker concentrations of electrolyte. Two curves of the P.D. of a red cell suspension in a series of electrolyte concentrations can therefore be recognized. A transient "immediate curve" in which the full effect of electrolyte is observed, which corresponds in type to the curves established by other investigators for several different types of particles, and a "final curve" in which that part which lies before the isoelectric point in the weaker concentrations of the series, and which shows the negative charge of the cells, has fallen to zero. In the stronger concentrations, showing the positive charge after reversal, the "final curve" coincides with the "immediate curve." The recognition of the different conditions represented by these two curves is essential for a proper understanding of the effects of electrolytes on the charge of the red blood cells. The "immediate curve" must be considered if the effects of electrolytes on the charge of the cells are to be fully demonstrated and compared with similar effects on other particles, such as collodion particles. The "final curve" is the one that must be correlated with other phenomena which develop in the standing suspension; notable among these are variations in stability with different electrolyte concentrations.

A correlation of the two curves, "final curve" of P.D. and curve of stability of the suspension, shows that, excepting in the highest concentrations of electrolyte, whenever the potential of the cells is above a certain critical value stability occurs. In the pure sucrose suspension the fall in P.D. of the cells which occurs as the suspension stands is accompanied by agglutination and instability. With trivalent and strongly hydrolyzing bivalent salts the strong positive charge which occurs after reversal is accompanied by a zone of stability; and when this positive charge is again depressed in the higher concentrations of electrolyte, instability again ensues. With bivalent salts which hydrolyze less, and so do not produce a positive charge which surpasses the critical value, this intermediate zone of stability is not found. A red cell suspension therefore resembles the oil droplet

suspension (Powis), the bacterial suspensions (Northrop and De Kruif), and the suspensions of collodion particles (Loeb), in that it shows a critical flocculation potential. There is considerable variation in the absolute value of the critical potential found by different investigators, and this variation does not seem to depend on the chemical nature of the suspended particles as each investigator has found the same value for particles of different composition. Powis and Ellis found a critical P.D. of 30 mv. for oil droplets and colloidal arsenous sulfide, while Northrop and De Kruif obtained a value of 16 mv. for bacterial suspensions and Loeb a similar value for collodion particles. Our value for collodion particles and red cells corresponds with that determined by Powis and Ellis.

The curve of "immediate charge" is not, however, without importance for the stability of the suspension, for if the velocity of the development of instability in the series of electrolyte concentration is closely observed under proper conditions, it will be seen that with those electrolytes which produce extremes of negative and positive charges (Fe and Al), aggregation of the cells occurs first in those mixtures in which the immediate charge has been depressed to the neighborhood of the isoelectric point, and that agglutination develops later in the other tubes as the equilibrium of the "final curve" is reached. In this zone of electrolyte concentration the electrolyte is the direct cause of the rapid agglutination of the suspension; elsewhere agglutination is due to the failure of the electrolyte to stabilize the suspension. One may speak of "rapid agglutination" in the region of the isoelectric point and of "slow agglutination" in those regions where the charging action of the electrolyte was insufficient to stabilize. This "slow agglutination" is well shown in the case of the bivalent salts $FeCl_2$ and $CdCl_2$. There is more extensive agglutination with them than occurs with trivalent ions, as all the tubes are agglutinated, but the agglutination is due to a lack of charging activity of the ions rather than to any "marked" action on their part as might be supposed by the extensive range of the agglutination.

An analogous condition in the flocculation of colloid solutions by electrolytes has been studied by Zsigmondy (13), who distinguishes two types of flocculation, a rapid and a slow. In a review of this work Freundlich (14)[2] discusses the pos-

[2] Freundlich (14), p. 592.

sible causes for the occurrence of the rapid and slow flocculation, and suggests that the transition from the slow to the rapid type may depend on the electrical properties of the particles. He states

"Man muss demnach vor allem das Konzentrationsgebiet der *raschen* konstanten Koagulationgeschwindigkeit von dem der *langsamen* veraenderlichen Koagulationgeschwindigkeit sondern. Es ist sehr wahrscheinlich dass die Grenze zwischen den beiden Gebieten, oder richtiger, der verhaeltnismaessig schnelle Uebergang von dem einen zu anderen, wiederum mit elektrischen Eigenschaften der Micellen zusamenhaengt. . . . Betrachtet man in diesem Zusamenhang die Kurve (curve of P.D.) so wird man jetzt zwei kritische Potentiale bzw. zwei kritische Ladungen zu unterschieden haben; das frueher schon eroerterte erste kritische Potential, das ereicht werden muss, damit die Flockung deutlich stattzuhaben beginnt; dann eben das zweite kritische Potential unmittelbar beim Nullpunkt bei dem die langsame Koagulationsgeschwindigkeit in das rasche uebergeht."

The occurrence of "rapid agglutination" in the neighborhood of the isoelectric point and of the development of "slow agglutination" in other parts of the P.D. curve is demonstrable with suspensions of red cells and trivalent ions, and Freundlich's theory is therefore confirmed for these conditions.

Another type of stability which is unrelated to the electrical properties of the suspended cells is also seen in our experiments. This is the occurrence of a "stable zone," best shown in the highest concentrations of non-hydrolyzing salts with bivalent and monovalent ions. It also occurs with other ions, but is in most cases obscured by the hemolysis which occurs in the high concentration of these salts. It is the same type of stability described by Northrop and De Kruif with these same salts and bacterial suspensions, and which they showed to be due to a depression of attractive forces (cohesion) between the bacteria. We have not studied this force in our experiments as the procedure used by these investigators to determine the degree of cohesion would completely denature the red cells. It is obvious that this zone of stability cannot depend on the electric charge of the red cells, for this is almost completely removed at the concentration in which the phenomenon is observed. Since the conditions of the red cell suspension resemble those of Northrop and DeKruif's bacterial suspension, it may be assumed for the present that the cause of the stability is the same in both.

Under the general head of stability of suspensions must also be considered the questions of "prozones" and "irregular series," first

described by Bechhold (15) and by Neisser and Friedemann (16). These phenomena have been intensively studied by Buxton, Teague and Schaffer (17). The latter authors distinguish between "irregular series" and "prozones," and the later work of Northrop and De Kruif shows that under certain conditions such a distinction is valid, for the mechanism of the production of the two phenomena may be different. The same relations hold true in red blood cell suspensions. In the case of Al_2Cl_6 both an "irregular series" and a "prozone" are present, and the differences in the mechanism of production are well illustrated. The zone of stability in the middle of the series ("irregular series") is the result of the strong positive charge of the cells; the zone of stability in the highest concentrations of the electrolyte ("prozone") may be assumed to be due to the effect of the electrolyte on cohesion, for it cannot be the result of the P.D. of the cells, which is low at this point.

The "prozones," which are observed in strong concentrations of $BaCl_2$, $CaCl_2$, and NaCl, likewise cannot be due to the P.D. of the cells and may be explained by variations in cohesion. All "prozones" are not, however, to be explained by this hypothesis, since the "prozone" which occurs in high concentrations of disodium arsphenamine is more likely the effect of the high negative charge which this alkaline solution produces on the cells. Nor can it be stated that "irregular series" are produced by all trivalent salts and by them alone, for we have shown that they are produced by strongly hydrolyzed bivalent salts like $CuCl_2$; and we will show in a later paper that they may be absent with trivalent ones. It is not the valency of the salt but the degree of positive charge following reversal that determines the "irregular series," and this may be the result of H ions in the solution.

The action of disodium arsphenamine on the red cell suspension merits special discussion. In a previous study (8) we suggested that the agglutination might be due to changes in the electrical properties of the cells, and the experiments reported in this paper show this to be the case. The curve of alterations in the charges of the cells and that of the stability of the suspension go hand in hand, and the relation resembles exactly that found in the action of arsphenamine on the suspension of collodion particles. Considering the series of mixtures from the weakest to the strongest concentrations, two effects of this

agent may be observed: an agglutinative and a stabilizing action. In the weakest concentrations no effect is noted and the cells agglutinate spontaneously as their negative charge slowly falls. From M/32,000 to M/8,000 "rapid agglutination" occurs, a direct result of the action of the arsphenamine which has depressed the P.D. of the cells below the critical value. From M/4,000 onward stability is present, and also the direct effect of the alkaline arsphenamine, which produces a high negative charge on the cells.

As has been shown in our former study (8) the presence of another electrolyte (NaCl) in sufficient concentration markedly modifies the occurrence of agglutination. Agglutination in 0.9 per cent NaCl suspension is quite different from that observed in an electrolyte-free sucrose suspension. From what we have seen of the action of NaCl on the properties of red cells it is evident that the selection of "physiological salt solution" as a suspension fluid for red cells in experiments on agglutination is a particularly unfortunate one, as with such a concentration of this electrolyte both the P.D. of the cells and the attractive forces (cohesion) between them are profoundly modified.

Since this paper was completed Northrop and Freund (18) have published the results of their studies on the agglutination of red blood cells. As is the case with suspensions of collodion particles, we find a critical potential for the blood cell suspension which is much higher than the value (8 to 10 mv.) they found, although the difference in the actual value of the potentials of the particles of both suspensions with the varying electrolyte concentrations is not so marked. This difference may perhaps be explained by a phenomenon which Northrop and Freund describe in the same article, namely that the value of the critical potential may be considerably altered by the presence of various substances in the suspension. Our suspensions, both of cells and collodion particles, may well have differed from theirs in the actual constitution as a result of different methods of preparation. The essential fact of the matter, concerning which there is no disagreement, would, therefore, seem to be that a critical potential exists for the agglutination of red blood cell suspensions and that the value of this potential varies with conditions in the suspension.

CONCLUSION.

Two types of stability are observed in suspensions of red blood cells. In weak concentrations of electrolytes the stability depends on the electric charge of the cells and suspension is unstable below a certain critical P.D.

In strong concentrations of electrolyte, the stability bears no relation to the charge.

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ON THE CRITICAL THERMAL INCREMENT FOR THE LOCOMOTION OF A DIPLOPOD.

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(Accepted for publication, August 7, 1924.)

I.

A good deal of attention has been paid to the temperature coefficients of vital processes. The data available up to 1914–15 are for the most part collected in Kanitz' (1915) book. The chief interest of this work has centered about the "R. G. T." (van't Hoff) rule. In common with many chemical reactions, at ordinary temperatures the velocities of a variety of protoplasmic activities are found to be about doubled or trebled by a 10° rise of temperature.

The temperature coefficient, Q_{10}, giving the ratio of velocities for an interval of 10°, is a quite imperfect means of characterizing a process. It is in any case not a *constant* quantity, but depends upon the particular temperature. Moreover, it is known that in the neighborhood of certain temperatures, varying with the process considered, abrupt changes occur in the temperature relations of many biological phenomena. A means is required of dealing with these "critical points." Such complexity of dynamical balance as must be postulated for living matter leads one to expect that by considerable change of temperature a different fundamental reaction might be brought into control of the velocity of a given phenomenon.

For certain vital activities which may be taken as typical, it is found that the underlying or controlling processes may be treated as systems of "irreversible" first order reactions (Osterhout, 1914, 1922; Hecht, 1918–19, 1918–19, *a*; Osterhout and Haas, 1918–19).

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To such processes the equation of Arrhenius (1889) should be applied. Originally with the status of a useful empirical relation, the theoretical foundation for this equation seems now quite extensive (J. Rice, 1915; Rodebush, 1923; Thomson, 1924). It considers that the velocity of an irreversible reaction is proportional to the exponential of $-\frac{E}{RT}$, where R is the gas constant, T the absolute temperature, and E the gram molecular energy of activation or "critical increment" of the "active" substance (or, for a bimolecular reaction, the sum of these energies).

As ordinarily employed, this relation has the form

$$K_2 = K_1 e^{\frac{\mu}{2}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)},$$

K_1 and K_2 being the velocity constants at the respective temperatures. The quantity μ $(=E)$ is independent of the temperature, and independent of the actual velocity of the process. For simple chemical processes μ varies in different cases from about 4,000 to 35,000. It is therefore a more delicate, as well as being theoretically a more significant, index than the Q_{10} ratio for the characterization of a process. The quantity μ, in distinction to the "temperature coefficient," might be termed the *temperature characteristic*.

Since μ has the meaning "energy of activation," it is to be expected that processes governed by the same species of "active molecule" will yield the same value of the constant μ. Thus, various reactions determined by the same catalyst should yield a practically constant "critical increment," namely that associated with the formation of active from inactive molecules of catalyst. This view has been developed in a very suggestive recent paper by F. O. Rice (1923), with particular reference to hydrogen ion catalysis. Reactions in living matter must frequently, if not usually, be conceived as catalytically excited. A preliminary survey has shown that vital phenomena are to a large degree empirically separable into groups according to the associated values of μ.

An illustration will serve to indicate the sort of additional information obtainable by means of the temperature characteristic. In a study of the toxicity of pure salts for the colonial protozoan

Zoothamnium alternans, Koltzoff (1913) measured the time required for disorganization ("cytolytic" collapse) of the "kinoplasm" of the contractile stalk at different temperatures, in 0.5 normal solutions of NaCl and of KCl. The reciprocals of these times give a measure of the velocities of the process underlying the disorganization. The observations are plotted in Fig. 1. It is clear that the value of μ in the Arrhenius equation is the same for each set of experiments, namely 16,680. This value of μ I find associated with certain oxidative processes (as in *Arbacia* eggs; data of Loeb and

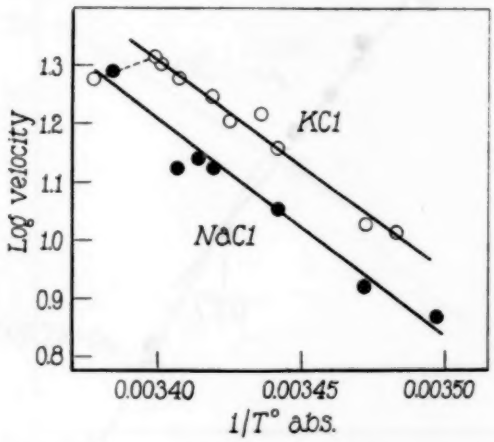

FIG. 1. The relation between temperature and the velocity of "cytolytic collapse" of the "kinoplasm" of the stalk of *Zoothamnium* in 0.5 N solutions of KCl and NaCl; data of Koltzoff (1913).

Wasteneys, 1911). The velocity with which carbon monoxide displaces oxygen from combination with hemoglobin, as given in the beautiful experiments of Hartridge and Roughton (1922–23), I find to yield μ = 16,525. The same value may be demonstrated also in the measurements published by Gray (1923–24); though here (as is not infrequently the case), at temperatures greater than about 15° a second process, in this instance with μ approximately 11,000, becomes dominant (Fig. 2). Gray's data give measures both of oxygen consumption and of ciliary activity in the gill epithelium of *Mytilus*. The second process indicated by Fig. 2 may be an

alkaline catalysis (*cf.* O. F. Rice, 1923); a value of μ probably identical with this is recognizable in other cases of tissue respiration (over a portion of the temperature range), for example in the figures given by Evans (1923), plotted in Fig. 3.

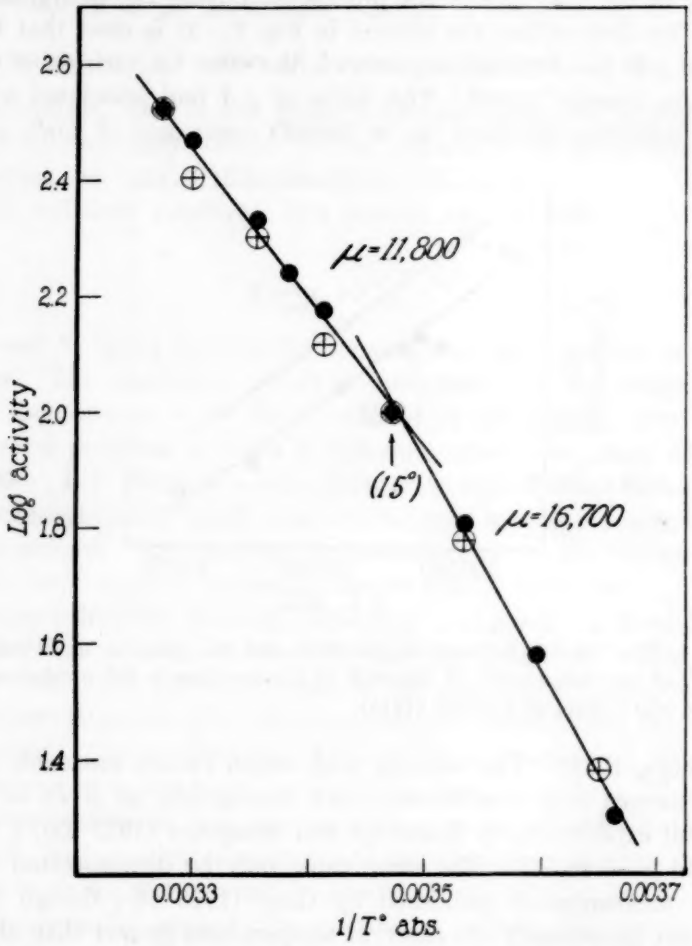

FIG. 2. Ciliary activity (black circles) and oxygen consumption (white circles) of *Mytilus* gill epithelium; data of Gray (1923). The two series of observations have been adjusted to give equal velocities at the mid-point (15°) of each set. At the higher temperatures the line for the oxygen consumptions is perhaps little lower than that for activity, but is in any case parallel thereto.

Loeb (1896) had observed that deprivation of oxygen leads to cytolytic destruction, and (1909) that the cytolysis of *Strongylocentrotus* eggs by NaCl could be suppressed by NaCN. It is known that pure salts affect the rate of respiration (Osterhout, 1919–20; Brooks, 1919–20), under conditions such that injurious action is involved. The conclusion, therefore, is consistent, that the toxic action of NaCl and KCl as observed in Koltzoff's experiments involves an effect on oxidations which determine the velocity of the toxic process as influenced by temperature.

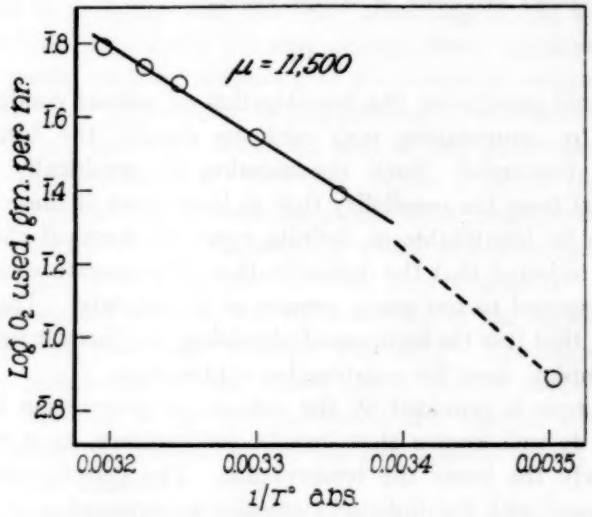

FIG. 3. Oxygen consumption of guinea pig uterus at different temperatures; data of Evans (1923). At some temperature between 25° and 12.5° the relative oxygen utilization is abruptly decreased.

Considerations of this sort may not infrequently permit decision as to whether physical alteration of the protoplasm (*e.g.* viscosity change) at particular temperatures is the *cause* of chemical disorganization, or itself a consequence of the net effect of temperature upon a system of linked reactions.

Scrutiny of much published data, and of newly investigated processes, shows that a "break" in the value of μ (such as that illustrated in Fig. 2) is not at all uncommon. Deviations at supranormal temperatures (*cf.* Hecht, 1918–19, *b*), and at very low temperatures,

are of course well known, and in favorable instances they may be analyzed. Abrupt change in the temperature relation indicates that a separate process has become the governing reaction in the complex. The occurrence of such alterations at about the "normal" temperature of an animal's environment is very important for the analysis of fluctuations in natural behavior (*cf.* Crozier and Federighi, 1923), as I shall endeavor to show in detail elsewhere. Here it is sufficient to point out that for the understanding of such complex temperature relations, observations of some precision are required at short intervals of temperature.

II.

From this standpoint the investigation of animal conduct as influenced by temperature may seek to classify the fundamental processes concerned. Such classification is empirically justified, quite apart from the possibility that at least some of these processes may then be identifiable as definite types of chemical change. It might be objected that the determination of temperature coefficients may be exposed to too many sources of uncertainty. The efficient answer is, that it is the business of physiology to discover regularities, and to employ them for constructive explanation.

An example is provided by the velocity of progression in arthropods. It is well known that insects, for instance, as a rule creep more slowly the lower the temperature. The velocity of creeping was measured with the diplopod *Parajulus pennsylvanicus* at temperatures between 6° and 30°. One series of the observations was secured by the cooperation of Mr. R. Forer and Mr. A. F. Mangelsdorf. Each animal was allowed to creep over a measured distance on a moist filter paper surface. A possible effect of light was looked for in control experiments, and found to be negligible. The size (length) of each individual (10 to 30 mm.) was recorded. The time occupied by the creeping act was measured with a stop-watch.

When the animal has recently emerged from quiescence creeping tends to be continuous and exactly in a straight line. The inner mechanism maintaining straightness of the creeping path has been discussed previously (Crozier and Moore, 1922–23). Particularly at and above a certain temperature a diplopod instead of smoothly

moving ahead may seem to "hesitate," and the rate of progression then becomes slow and uneven. This type of irregularity, when apparent, tends to become obvious at about 15°. For present purposes all such irregular creeping has been excluded. Another and quite interesting type of "irregularity" is the apparent inability of some individuals to show speedier progression than that evidenced at about 15°; for these individuals, the rate of progression is practically constant at temperatures between 15° and 30°. The records of these animals have been excluded.

The rate of creeping does not vary according to the size of the animal, within the limits of size among those employed. This probably means that the efficiency of the locomotor organs, chiefly a matter of their dimensions, is proportional to the animal's bulk; such a relation has been indicated also in the gasteropod *Limax maximus* (Crozier and Pilz, 1923–24), where a given increase in frequency of pedal waves produces at the same temperature an increase in velocity of vertical creeping which is independent of the animal's weight (except perhaps for very large animals).

The creeping act involves the rhythmic use of the approximately 58 pairs of legs, two pairs to each "segment." The opposite legs forming a morphological pair move synchronously and in phase; the phase relations of the movements of the successive pairs are such that about 6 "waves" of moving legs appear on the animal's length (*cf.* also Lankester, 1904).

The observations are collected in Fig. 4. Two series of measurements, made respectively in early spring and in June, have shown satisfactory agreement. It will be observed that in the neighborhood of 16° the observations show minor irregularity.

The velocity of uniform creeping varies with temperature according to the Arrhenius equation. The curve drawn in Fig. 4 is the curve of this equation with the critical increment put $\mu = 12{,}245$.

III.

The speed of creeping of ants has been measured at various temperatures by Shapley (1920). To his extensive observations Shapley fitted an empirical formula. Fig. 5 shows that over what may be considered the "normal" range (16° to 38.5°) the speed of creeping

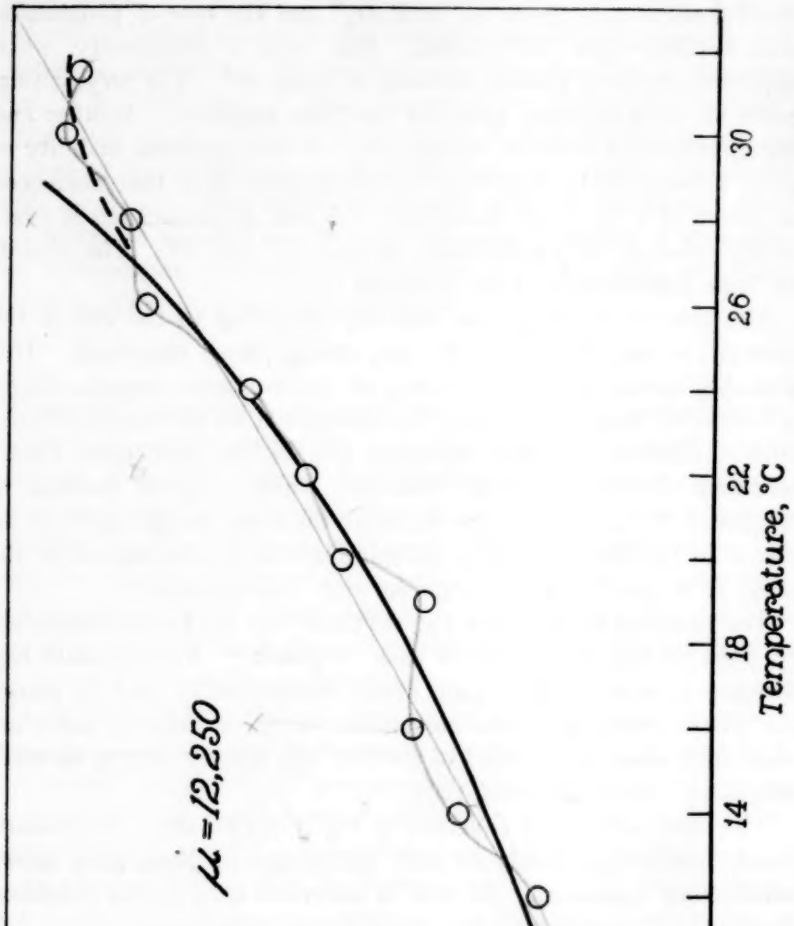

FIG. 4. The velocity of progression of *Julus* as function of temperature. The curve is the graph of the Arrhenius equation. Deviations appear below 8° and above 28°. The irregularity near 16° is commented upon in the text.

varies in logarithmic relation with the reciprocal of the absolute temperature, μ being = 12,220.

Of the various elements entering into the act of locomotion we may suspect that some central nervous phenomenon akin to "nerve center discharge" plays a controlling part. It is possible to show that nervous interconnections exist between the limbs of arthro-

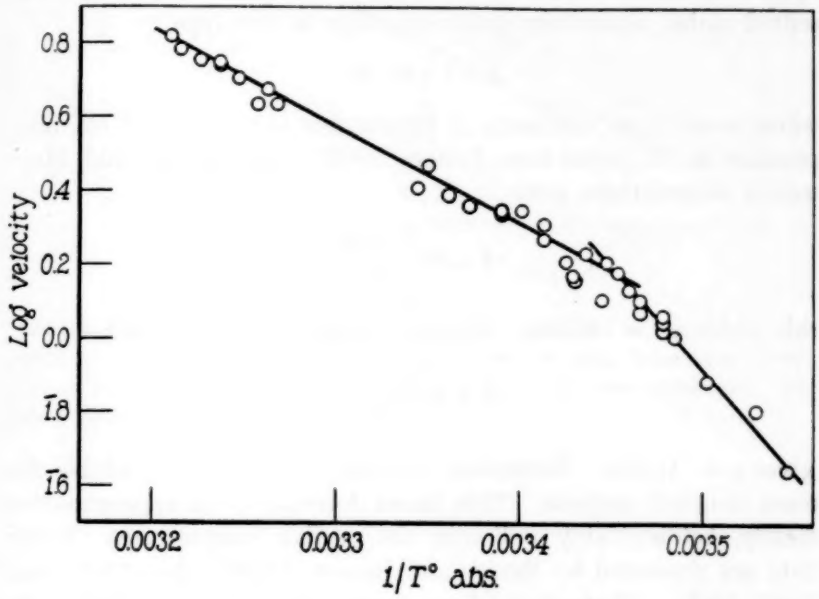

FIG. 5. The velocity of creeping of the ant *Liometopum apiculatum* is plotted logarithmically against the reciprocal of the absolute temperature (data of Shapley, 1920). Between 16° and 38.5° the value of μ = 12,220; below 16°, 25,900.

pods, such as must imply "nerve center" activity in their rhythmic use. The action of atropine, in the case of caterpillars, converts protrusion of the legs and their locomotor action into retraction when the usual local stimulus to creeping is applied (Crozier, 1922). The displacement of one leg of a *Ranatra* in a state of maintained tonic immobility causes characteristic movements of its mate (Crozier, 1923).

With the supposition that the rate of movement of the legs of *Parajulus* and of ants depends upon the rate of discharge of impulses from nerve centers, we may look among arthropods for other phenomena in which the rate of central nervous activity is likely to be a governing element.

1. It has been known for some time that the frequency of "calls" by the "common cricket" (? *Œcanthus*) and by the katydid is represented rather accurately by an equation of the type

$$a\,T + b = N,$$

where a and b are constants, N the number of calls, and T the temperature in °F. (cited from Folsom, 1906[1]). For the katydid, Hayward's observations gave

$$T = 60\,\frac{N - 19}{3};$$

this yields $\mu = 12{,}500$. Dolbear's (1897) formula (cricket),

$$T = 50\,\frac{N - 40}{4},$$

gives $\mu = 11{,}300$. Numerical observations are not available for more detailed analysis. This linear formula is an approximation merely, holding fairly well over the median temperatures. Fuller data are presented by Bessey and Bessey (1898), Edes (1899), and Shull (1907). These data I have averaged graphically, reducing the temperatures to °C., and the results are given in Fig. 6. It is apparent that the critical increment is in each case near 12,000. Of the several sets of observations that set by Shull is the largest and best (particularly with regard to the accuracy of the temperature measurements); the value of μ is 12,200.

2. The frequency of flashing of fireflies as a function of temperature has been considered by Snyder and Snyder (1920). From their data (Fig. 7) $\mu = 12{,}200$.

3. That this value of the critical increment does not reflect merely a relation between temperature and capacity of muscle fibers to

[1] Folsom (1906), p. 106.

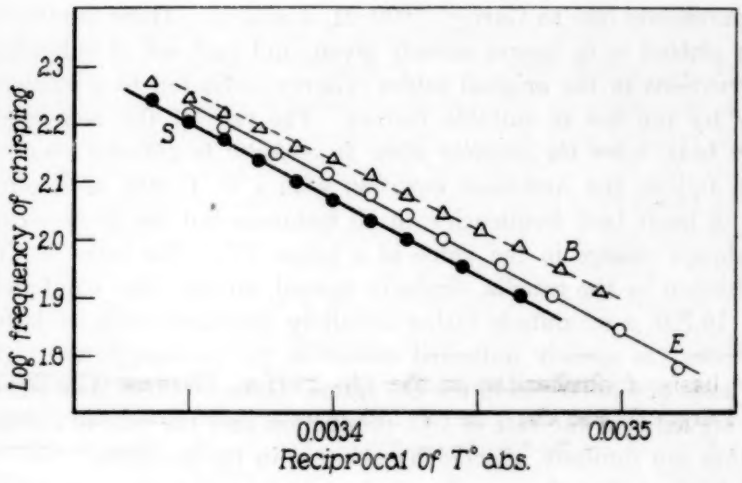

FIG. 6. The rate of chirping of the tree cricket *Œcanthus* as function of temperature; *B*, data from Bessey and Bessey (1898); *E*, data from Edes (1899); *S*, data from Shull (1907); the last series is probably the most significant. The observations were all made in the field.

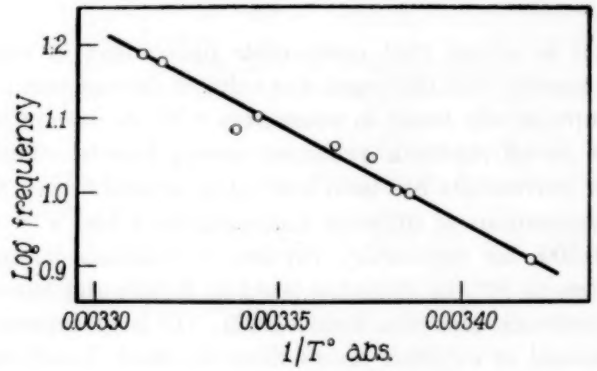

FIG. 7. The frequency of flashing of fireflies as fu nction of temperature (Snyder and Snyder, 1920); the critical increment is $\mu = 12{,}200$.

respond to impulses is definitely shown by an important series of measurements due to Garrey (1920–21, *a* and *b*). These data have been plotted as in figures already given, and each set of individual observations in the original tables (Garrey) adjusted to a common scale by the use of suitable factors. The rate of the neurogenic heart beat, *when the ganglion alone is subjected to different temperatures*, follows the Arrhenius equation with $\mu = 12{,}000$, as is often true of heart beat frequencies; in all instances but one there occurs an abrupt change in the value of μ below 15°. The rates of CO_2 production by the ganglia, similarly treated, on the other hand yield $\mu = 16{,}700$, a magnitude rather definitely associated with oxidative processes (as already indicated earlier in the present paper). On the basis of similarities in the Q_{10} ratios, Garrey (1920–21, *a* and *b*) had argued that the rate of CO_2 production and the rate of cardiac rhythm are similarly affected by changes in temperature.

It is clear that these various processes in which the rate of activity of "nerve centers" is involved do provide closely concordant values of the critical increment. A common chemical basis of definite type may therefore be provisionally assumed for them. To what particular structural element the scene of this chemical process may be allocated is of no special consequence for the present, nor can it be definitely stated to what sort of reaction the process belongs.

It should be added that comparable phenomena in vertebrates do not necessarily yield this particular value of the constant μ (though it is not infrequently found in connection with the rate of the heart beat), nor do all rhythmic processes among insects. The rate of respiratory movements has been studied in several forms, and from series of estimations at different temperatures I find μ = approximately 16,500, for respiratory rhythm in *Libellula* larvæ (up to 15°) and (up to 30°) in *Dixippus* (data of Babák and Rŏcek, 1909; von Buddenbrock and von Rohr, 1922). It is of interest that a certain amount of evidence shows these rhythmic breathing movements to be to some extent governed by the oxygen tension; this point should be more thoroughly investigated.

SUMMARY.

A comprehensive theory of animal conduct must be in position to account for variability in behavior. To some degree this may be possible through the quantitative examination of alterations in behavior as determined by temperature. This idea requires for its development a means of classifying vital activities on the basis of their "temperature characteristics." Such basis is provided by the critical increment (μ) in Arrhenius' equation for the influence of temperature upon the velocity of irreversible reactions. There is also obtained in this way a method of dealing with singular points at which the relationship between temperature and activity changes, and at which behavior tends to be especially variable.

As an example of such classification, the critical thermal increments are computed for the rate of locomotion in the diplopod *Julus* and in the ant *Liometopum*. These are found to be identical, μ being respectively 12,250, and 12,220, and to agree quantitatively with the critical increments obtained for certain other types of rhythmic activity (among arthropods) in which the rate of "central nervous discharge" may be assumed to play a governing rôle.

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CRITICAL THERMAL INCREMENT FOR THE MOVEMENT OF OSCILLATORIA.

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(Accepted for publication, August 7, 1924.)

I.

The characteristic movements of the filaments of the blue-green alga *Oscillatoria* have not uncommonly been held due to the activity of a superficial layer of substance, a kind of "plasma membrane," external to the cell wall (*cf.* Pfeffer, 1906; Schaeffer, 1920). Other theories as to the mechanism of these movements have invoked "osmotic processes" between the cells and the medium (*cf.* Vines, 1886), or the secretion of mucilaginous substance over all or part of the surface of the filament (*cf.* Correns, 1897; Gicklhorn, 1921).

This type of motility is of interest in itself, and also for the information it may perhaps be made to give as to the nature of the movements of slender protoplasmic processes. Measurements of the temperature coefficients of ameboid progression, for example, or of phagocytosis (Fenn, 1922), do not necessarily have a direct bearing on the interpretation of motion in pseudopodial projections, or in such structures as the terminal arborizations of neurones (if it be that in fully developed nervous organs these processes do indeed undergo retractions and protrusions). Some more recent notions (Schaeffer, 1920; Mast, 1923) as to the basis of ameboid creeping depend upon the activity of a "fluid outer surface layer," demonstrated by the streaming movement of adhering particles (Bütschli, 1892; Gruber, 1912), and identified by Schaeffer (1920) with the "plasma membrane." The nature of the activity of this superficial protoplasm is quite obscure (*cf.* Chambers, 1924). *Oscillatoria* seems a most suitable organism wherewith to investigate it.

It may be expected that the superficial streaming motion revealed by attached particles is not very dissimilar in *Oscillatoria* (Siebold and Schultze, as cited in Pfeffer, 1906; Schaeffer, 1920), in diatoms (Müller, 1889, 1897; Lauterborn, 1896), and in at least some amebæ.

With a properly selected species, the rate of movement of *Oscillatoria* becomes a delicate index of the effects of temperature and of certain other agencies. Several aspects of the motility are susceptible of measurement. In some species the free trichomes swim in spiral path through the water; with other forms this type of motion is not so pronounced. Again, if one end of a filament is held in a colonial mass, the free portion may move in a conical path. Under constant conditions, the rate of this activity is very steady, as we have found. With certain species a gliding motion in contact with glass or other surface is most conspicuous. In uniform environment the rate of such translatory movement is remarkably constant. It is well known that the direction of the motion suffers periodic reversal (somewhat as in an Ascidian heart), at which times the direction of spiral streaming on the outer surface of the filament is said also to be reversed.

Neither in the particular species used for our measurements nor in two others available for observation have we been able to convince ourselves that the filament as a whole rotates during the translatory motion. Observations with ordinary microscopic equipment were supplemented by the use of dark-field illumination and an immersion objective. The pigment granules are easily followed. There is not more than a barely detectable layer of "slime" coating a filament, which itself does not visibly rotate. There is no appreciable protrusion of slime at either end of a filament during active motion, nor a detectable alteration of the surface or the ends of the filament when the direction of movement is reversed. This was verified by study of filaments in suspensions of Chinese ink. An occasional jerky movement, probably due to contact of the advancing filament with some obstacle, may rotate the filament slightly. When the filament rubs over a bit of debris there is no obvious disturbance of the surface such as might be visible if "slime" were present. The fact that the rate of movement is independent of the length of the filament would seem to argue against the theory that the

end-cell is a locomotor organ. We have observed that under dark-field illumination the protoplasts of a moving filament seem to contract rhythmically, but the significance of this fact has not been fully made out.

II.

For the present inquiry we have utilized the linear translatory movement of a species of *Oscillatoria* in which this sort of motion is well developed, and in which the filament as a whole does not rotate upon its long axis when in contact with glass.

Our cultures were started with "film" from the surface of a sewage-purification filter-bed, in which two species of *Oscillariæ* were abundantly represented. We are indebted to Dr. Margaret Hotchkiss for a supply of this material. In shallow water at the bottom of a covered crystallizing dish the plants may be maintained in good condition for a long time. All the observations were made with one species, which we have not been able to identify with certainty; the preparations were made from a single culture dish, kept in diffuse light, in which the pH remained practically constant at 7.4. The filaments of this species are 0.0034 mm. wide and in length vary between 0.1 and 1.2 mm.

The filaments were removed in small quantities from the culture dish with the aid of a wide mouth pipette and mounted under a supported cover-slip. The slide was placed in an electrically heated warm stage with thermostatic control; the objective of the microscope passed into this chamber through a flexible ring of rubber cemented to the cover. The thermometer bulb was close to the preparation. For lower temperatures a similar box was prepared large enough to accomodate a small brine coil. The whole apparatus was also enclosed within a large glass chamber cooled with ice. Illumination practically constant was obtained with a substage lamp.

A series of the readings at low temperatures was obtained through the use of a cold chamber at the New Brunswick Laboratory of E. R. Squibb and Sons. The warm stage equipment, as set up in this refrigerated room, permitted readings at temperatures down to 6°C. For courteously allowing us to use the facilities of their plant in this way we are indebted to Dr. Lloyd K. Riggs of the research department of Squibb's.

The velocity of the moving filaments was determined with a 4 mm. objective and 8 × ocular, at tube length 151 mm. Each division of the ocular micrometer then corresponded to 0.0340 mm. At higher temperatures the time required to traverse three of these divisions was obtained in each case. At room temperature this time was about 25 seconds. Thus the movement is a slow one, 0.00408 mm. per second, but about ten times as fast as that of leucocytes under normal conditions. The low velocity made it easy to secure accurate estimations. At lower temperatures, the time to cover one micrometer division was taken, as the slow rate of movement might for longer distances of travel more often lead to the upsetting of readings through chance contact with other organisms in the preparation. The end of a moving filament was easier to follow, as a rule, than a definite pigment granule in a mid-region. The readings were taken from straight filaments in unimpeded motion. The time was read with a stop-watch. The temperature was constant within 0.1°C. or better for each set of observations.

The degree of consistency in the readings is illustrated in several sets plotted in Fig. 1.

Although the method of handling the filaments was as nearly as practicable the same in each preparation, we were led to examine the possible effect of mechanical agitation upon subsequent movement. This was tested by measuring the velocity of movement of filaments which had been shaken in a small amount of culture fluid. We desired particularly to get information on this point as a means of controlling subsequent experiments with salts. In one series of tests the material was placed in a small tube strapped to the bob of an electrically driven pendulum having a period of one second. The current through the actuating coil was so increased that at the end of each complete vibration the pendulum received a definite "bump," resulting in the forcible agitation of the algæ in the tube. With intervals of this agitation extending up to 30 minutes, no perceptible change was measurable in the velocity of movement. Irregular, but still gentle, agitation in a bottle for 3 minutes gave no effect at 29.6°C. Similar agitation for 6 minutes led to a slight acceleration of movement immediately after shaking (0.149 divisions per second as compared with 0.136

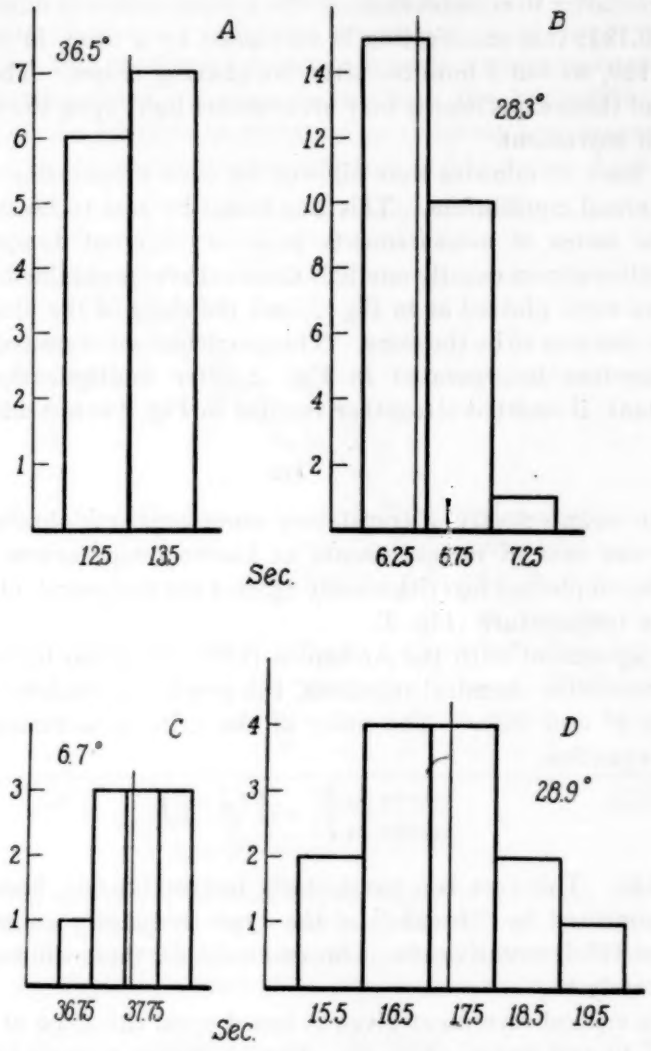

FIG. 1. Frequency distribution of several series of measurements giving the time, in seconds, required for different filaments of *Oscillatoria* to traverse 3 micrometer divisions (*A*, *D*) or 1 micrometer division (*B*, *C*).

The variation in these measurements is small, but too great to be explained as due to mere errors of timing. In each case the position of the mean is shown.

before); after 10 minutes shaking, the velocity observed immediately was 0.182; this acceleration is succeeded by a drop, in this case to 0.129, within 5 minutes after the shaking ceases. The extension of these experiments may give further light upon the mechanism of movement.

At least 10 minutes were allowed for each preparation to come to thermal equilibrium. This was found by test to be sufficient.

One series of measurements gave at different temperatures velocities almost exactly one-half those otherwise obtained. These figures were plotted as in Fig. 2, and the slope of the line fitting them was seen to be the same. This particular set of measurements is therefore incorporated in Fig. 2 after multiplication by a constant; if omitted altogether the line in Fig. 2 is not affected.

III.

The mean velocity of translatory movement was obtained from forty-one sets of measurements at known temperatures. These are shown plotted logarithmically against the reciprocal of the absolute temperature (Fig. 2).

In agreement with the Arrhenius (1889) equation for velocities of irreversible chemical reactions, the graph is a straight line (between 6° and 36°). The value of the critical increment, μ, in this equation,

$$\frac{\text{velocity at } T_2^\circ}{\text{velocity at } T_1^\circ} = e^{\frac{\mu}{2}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)}$$

is 9,240. This case is a particularly instructive one, because it is not confused by "breaks" of the type frequently encountered. Above 37° destructive effects become evident; these will be studied separately.[1]

The critical increment given is based upon the slope of the line fitted to the means (Fig. 2). The scattered means are closely confined (with one exceptional point known to be of doubtful

[1] The maximal temperature as obtained under the conditions of these experiments agrees fairly well with that given by Velten and others for cyclosis in cells of other plants (Davenport, 1897). It is well below the "upper thermal death point," which is above 40°C.

value) between two lines parallel to that giving the best fit. The reality of this fact is further demonstrated in Fig. 3, where the highest and the lowest readings in each set of measurements are similarly plotted. For a case in which the temperature coefficient of the latitude of variation at different temperatures is the same as the temperature coefficient of the mean, Loeb and

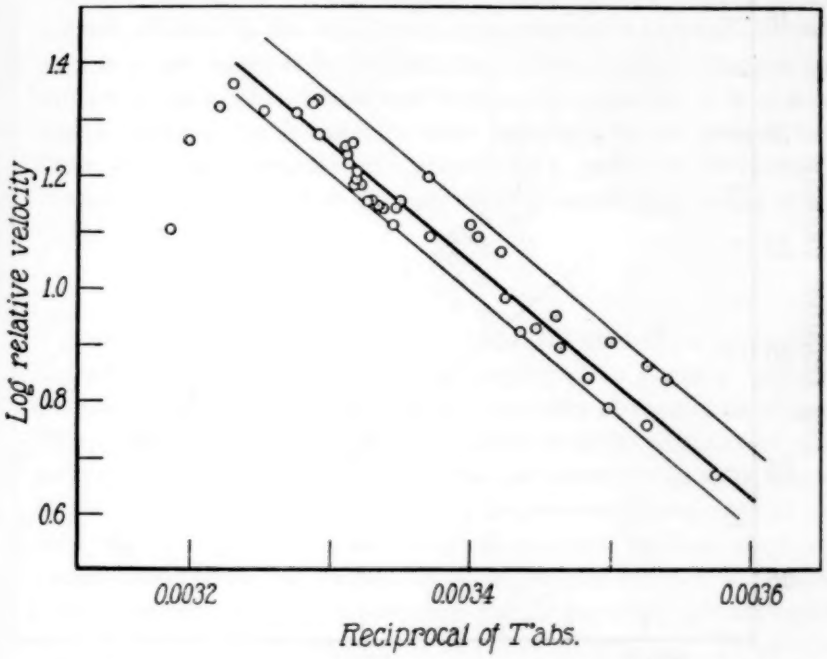

FIG. 2. The logarithm of the mean relative velocity of movement (100 times scale divisions per second) for each set of measurements is plotted against the reciprocal of the absolute temperature. The extreme variates from the best fitting line are themselves confined within two lines parallel thereto.

Chamberlain (1915) suggested that the variability might be due to the fact that in different individual organisms the effective amount of a catalyst for the process measured fluctuates within definite limits. The latitude of variation should thus be a constant fraction of the mean, over the temperature range considered. The type of graphic representation used in Figs. 2 and 3 gives a neater

method of demonstrating this point. Other similar cases might be cited (*e.g.* Crozier and Pilz, 1923-24).

The magnitude of the critical increment for the mean velocity of movement, and the fact that the extreme high and the extreme

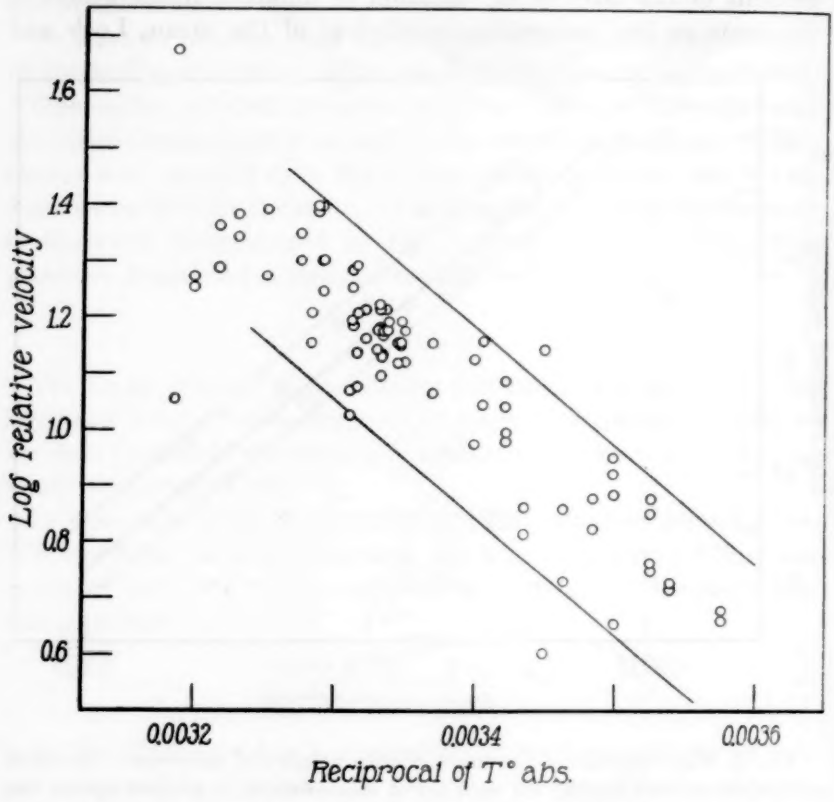

FIG. 3. For each set of measurements the extreme high and low variates are plotted as in Fig. 2. The latitude of variation is not lawless, but tends to be at each temperature a constant fraction of the mean. The slope of the confining lines is the same as that fitting the means of all the series (Fig. 2).

low variates at the different temperatures yield the same value of μ, are grounds for the conclusion that the translatory motion of this species of *Oscillatoria* depends upon the velocity of a chemical process. The velocity of this reaction is governed in charac-

teristic fashion by temperature, and the specific reaction rate seems proportional to the amount of some substance (catalyst) which in different filaments of the alga varies within definite limits (perhaps also in the same filament, from time to time).

This conclusion is quite independent of the actual mechanics of the movement. It concerns merely the type of activity which controls the speed of movement. Thus, surface tension effects are not evident in the relation to temperature, whatever rôle they may actually play; it is obvious that while viscosity changes and surface tension phenomena are frequently appealed to in physiological theorizings, it must in some instances be recognized that these physical properties are altered as a resultant of chemical change, rather than that they serve as a controlling cause of activity (Crozier, 1924–25).

IV.

It has been suggested (Crozier, 1924–25) that the magnitude of the critical increment (μ) may be employed to identify types of chemical reaction, especially among processes obviously catalyzed. This view is based in part upon empirical findings with varied vital activities, and upon a theory of temperature coefficients due to F. O. Rice (1923). While the very incomplete knowledge of critical increments for simple chemical systems perhaps does not permit more than the deduction of suggestive hints, it is possible from this standpoint to compare rather precisely physiological processes known or suspected to be really analogous. The critical increment for most chemical processes is above 10,000. Where diffusion is probably concerned, or some surface action analogous to that involved in the development of vapor tension, as in the action of trypsin on powdered casein or of powdered castor beans on cotton oil, the value of μ is near 7,500. To what particular group of processes the one fundamental to the motion of *Oscillatoria* may be assignable is not yet clear.

In seeking other physiological processes for comparison, we may for the present consider merely some activities not unlikely to be in a measure related. Among these, the "rate of loss of water" from certain plant cells (Delf, 1916) yields $\mu = 20{,}000$ (above

26°C.); this figure is derived chiefly from measurements of the rate of shrinkage of onion leaf (Fig. 4), corresponding data for dandelion scape (Delf, 1916) being found to have a curious discontinuity

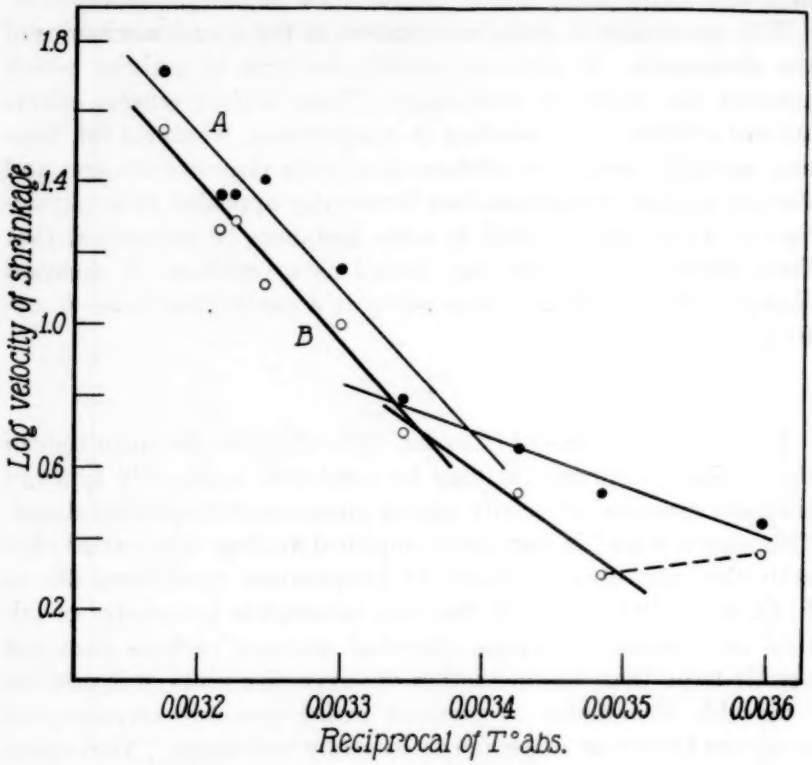

FIG. 4. The velocity of shrinkage of onion leaf bathed in 0.3 M sugar solution; *A*, at 30 per cent completion of the shrinkage process; *B*, at 50 per cent completion. Data from Miss Delf's (1916) tables. Above 26° the critical increment is μ = 20,000. The shrinkage measured was interpreted by Delf as due to loss of cell water by normal processes of permeability. Below 26° μ decreases to about 10,000, but the measurements are too irregular for analysis.

in the mid-range of temperatures. However, this is a slow process, and perhaps not one very pertinent to the discussion.

A connection has been suspected between protoplasmic streaming and the movements of *Oscillatoria*. The rates of cyclosis in

cells of several plants yield μ = 4,700 to 10,300, in the range of "normal" temperatures. (Fig. 5, based on Velten's observations; data reduced in Davenport, 1897, and Kanitz, 1915). So far as these critical increments serve as indication there is no very definite parallelism between cyclosis and the creeping of *Oscillatoria*.

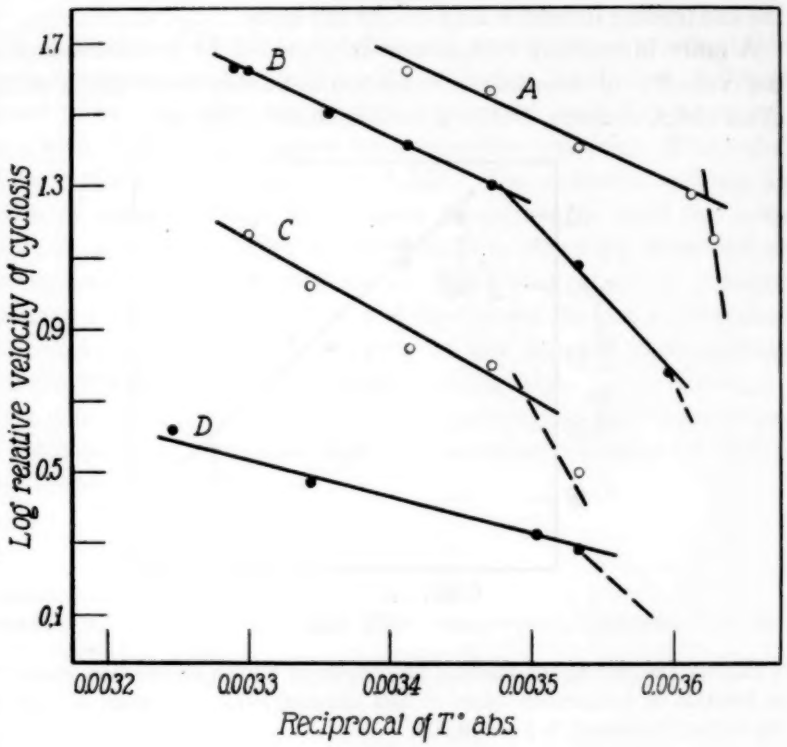

FIG. 5. The velocity of cyclosis in cells of several plants. The actual data (due to Velten; reduced in Davenport, 1897; Kanitz, 1915) have in each case been multiplied by a factor, which permits all four graphs to be shown in one figure. The measurements at higher temperatures and at very low temperatures are omitted. The lines show a definite change in thermal effect beginning somewhere near 15°C. (*A*, *Chara*, μ = 8,450; *B*, *Vallisneria*, μ = 8,450; *C*, *Nitella*, μ = 10,300; *D*, *Elodea*, μ = 4,780).

Scanty data on the rate of pulsation of the contractile vacuoles of *Infusoria* (*cf.* Kanitz, 1915[2]) lead to higher values of μ in certain instances, and in one case (*Euplotes*) to a lower magnitude (7,900). Where a phenomenon (such as pulsation rate in these vacuoles) is unquestionably determined by a number of concurrent influences, it is not unreasonable to expect that in different species the controlling influence may not be the same.

A more interesting comparison is provided by measurements of the velocity of ameboid progression in human neutrophilic leucocytes (McCutcheon, 1923); μ here is 10,800 (Fig. 6).

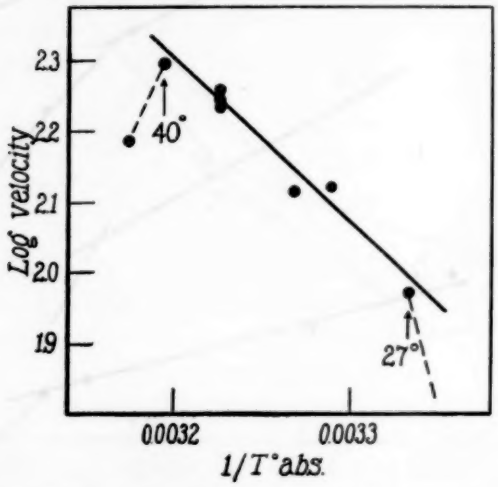

FIG. 6. The velocity of ameboid progression of human neutrophilic leucocytes as function of temperature (data of McCutcheon, 1923). Between 27° and 40° the critical increment is μ = 10,800.

These comparisons of course by no means involve the conclusion that the velocity of progression in leucocytes, for example, is determined by the activity of the "outer layer of protoplasm," as vaguely conceived in certain theories of ameboid movement. The critical increments indeed are in all probability significantly different. Pending more detailed investigation all that need be said is, that the velocity of cyclosis in certain cells, of creeping in leuco-

[2] Kanitz (1915), p. 62.

cytes, of pulsation in the vacuoles of some ciliates, and of translatory motion of *Oscillatoria* filaments, are all influenced in a not very dissimilar way by the temperature, although the actual values of the several critical thermal increments seem distinct and characteristic.

SUMMARY.

In a species of *Oscillatoria* exhibiting movement of type suitable for exact measurement the velocity of linear translatory motion is found to be controlled by the temperature (6 — 36°C.) in accordance with Arrhenius' equation for irreversible reactions. The value of the critical increment (μ) is 9,240. The extreme variates in series of measurements at different temperatures yield the same value of μ. The velocity of movement is therefore regarded as determined by the velocity of an underlying chemical process, controlled by the temperature and by the amount of a substance (? catalyst) whose effective quantity at any moment varies within definite limits in different filaments of the alga.

On the basis of its temperature characteristic the locomotion of *Oscillatoria* is compared with certain other processes for which this constant is calculated.

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PHOTOTROPIC CIRCUS MOVEMENTS OF LIMAX AS AFFECTED BY TEMPERATURE.

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(Accepted for publication, August 7, 1924.)

I.

The forms of the quanitative relationship between temperature and the responses of animals to light might be expected to differ according to the aspect of reaction which is measured as index of excitation. Thus in the clam *Mya* (Hecht, 1919–20, *a*) the primary photic excitation process for siphon retraction has a very low temperature coefficient such as is characteristic of photochemical changes, while the ensuing transformation occupying the sensory latent period shows (Hecht, 1918–19) a critical increment $\mu = 19{,}680$, which is typical of certain sorts of ordinary chemical phenomena.

On the other hand the velocity of movement of *Necturus* as result of illumination (chiefly or almost entirely of receptors in the skin) yields a low critical increment, $\mu = 7{,}770$ (calculated from Cole's measurements (Cole, 1922–23, *b*, see Fig. 1)). The low value of this temperature characteristic can scarcely be due to the latency or refractory period of the muscles involved; Bazett's figures (Bazett, 1907-08) show the critical increment for refractory period in the frog sartorius to be $\mu = 18{,}400$ (*cf.* Fig. 2), and Wolley's (1908) determinations of latent period and velocity of conduction in amphibian muscle yield increments, respectively, 10,980 and 19,120 (*cf.* Fig. 2). It is improbable that nerve conduction should be the limiting process in this activity; the increments for nerve fiber transmission are distinctly higher than 7,500; Lucas' (1908) determinations lead to values between 8,080 and 10,700; although the point cannot be gone into here it is possible to show that the temperature coefficients for velocity of

nerve conduction (Maxwell, 1907) reveal more than one participating process. It should be stated that in some of the papers referred to the graphs giving the effect of temperature are quite misleading,

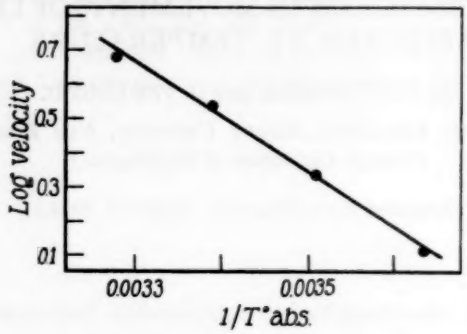

FIG. 1. The velocity of movement in *Necturus* exposed to light, as a function of temperature (data from Cole, 1922–23). The critical thermal increment is μ = 7,770.

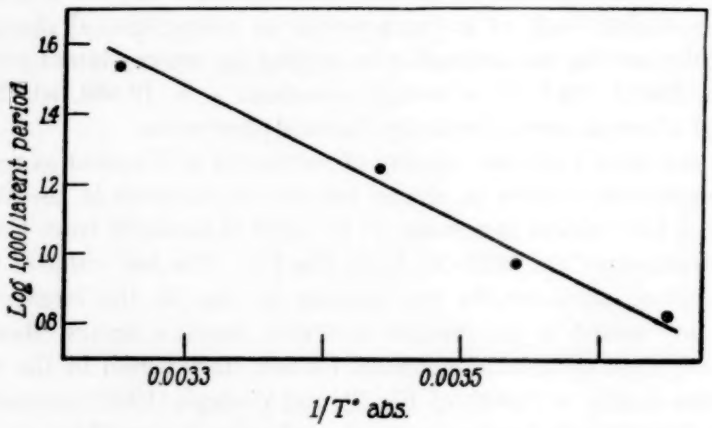

FIG. 2. The critical increment (μ = 18,400) for refractory period in frog sartorius (data from Bazett, 1907–08).

because *time occupied* by the process is considered, rather than (as should be the case) the *velocity* or reciprocal of this time.

A critical increment of this order (7,500 ±) is, however, associated with certain aspects of "nerve center" (ganglion cell ?) activity.

Cooper and Adrian (1924) have shown that when the frog spinal cord is cooled, the muscles remaining at room temperature, the distinctly larger variations in the electromyogram become fewer (although the frequency of total number of vibrations is sensibly constant); their interpretation is, that the number of ganglion cells which can discharge in unison is decreased by lowering the temperature. Piper's (1910) figures give for this frequency (in the neck retractor muscles of tortoise) μ = 7,400. The figures tabulated by Cooper and Adrian (1924) give μ = about 8,900. The critical increment obtained with the reaction of *Necturus* probably has reference to the activity of the

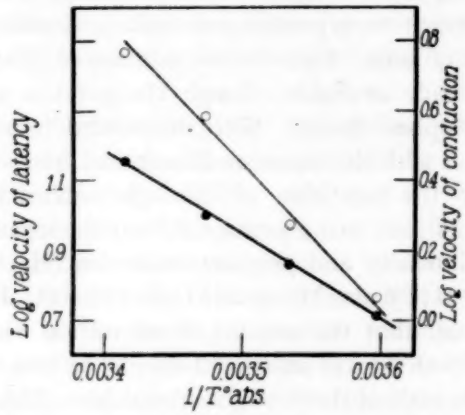

FIG. 3. Critical increments for latent period, μ = 10,980, and for velocity of conduction, μ = 19,120, in amphibian muscle (data from Wolley, 1908).

central nervous system, rather than to any property of the excitation process or of the musculature.

The general view-point to which such considerations as these may lead is important for the comprehension of phototropic behavior. It is probable that the relations of temperature to quantitative response may be used to analyze the connection between properties of the stimulus and the amplitude of reaction. The problem of the reversal in the direction of phototropic conduct which is in certain cases (Loeb, 1893; 1918) determined by lowering or by raising the temperature, is only remotely implicated. What is required is an understanding of the nature of the connection between the external stimulus and the amplitude of the induced orientation.

This matter is most conveniently approached through the consideration of "circus movements." But before measurements of phototropic circus movements can be applied to the prediction of the paths expected in normal orientation, it is necessary to know rather definitely the precise mechanism of the orientation process. Otherwise it is impossible, for example, to select the proper units in which to measure the circus movement effect. The few studies of circus movements thus far available (Minnich, 1919; Cole, 1922) do not fully satisfy this requirement.

The question involved is, at bottom, this: knowing in a particular case the orienting effect of light as exerted through each of two symmetrical photoreceptors, to predict quantatively the orientation induced by illumination of both. Data for the solution of this problem have not yet been made available, though the point is a fundamental one for phototropism theory. Since in several forms studied the orientation effect with the organism illuminated from one side only is proportional to the logarithm of the light intensity (Cole, 1922; Hartline, 1923–24), and hence perhaps reflects the logarithmic relation between light intensity and primary excitation (Hecht, 1919–20, *b*; 1922–23; 1923–24; *Cf.* also Moore and Cole, 1920–21), Hartline (1923–24) has suggested that the amount of orientation under differential illumination of both sides of an animal should be proportional to the logarithm of the ratio of the two light intensities. This would involve the assumption that the net result is an algebraic summation of two opposed orienting tendencies, without the intervention of any central nervous complication; in fact, a somewhat better agreement with observation is given by a different formula (M. M. Moore, 1923–24), which, however, has no obvious physical meaning. There is also involved a difference in units of measurement. Orientation under asymmetrical excitation has been computed in terms of degrees deflection per unit length of path (Minnich, 1919; Cole, 1921–22; Hartline, 1923–24), while amplitude of orientation under opposed differential beams has been measured as angle only (Patten, 1914; Loeb and Northrop, 1917; Northrop and Loeb, 1922–23). It is not clear that the two measures are necessarily commensurate. And in particular, full information has been lacking as to the two phenomena, circus movement effect and orientation under opposed lights,

so far as one animal is concerned. This information we (Crozier and Cole, 1922, *a*; 1921–22, *b*; 1923) expect to go some way toward supplying for the gasteropod *Limax maximus*.

II.

In dealing with phototropic circus movements of *Limax* (Crozier and Cole, 1923) it became important to know the relation between temperature and the differential muscle contractures on either side of the body. Asymmetrical tonus, continuously maintained, is responsible for the execution of a circular path when a *Limax* from which one eye-tentacle has been removed creeps under vertical illumination. The fact that the effector parts having to do with locomotion (the pedal surface) and with turning (parietal muscles) are totally separate from one another, makes it possible with such gasteropods (Arey and Crozier, 1921) to pay due observance to the necessary distinction between creeping and turning.

It was found by Crozier and Cole (1923) that in the performance of circus movements the actual amount of turning, measured in degrees of deflection per cm. of path pursued, is related to the velocity of creeping. A high velocity of linear progression is associated with a low expression of the turning tendency, while slow creeping is associated with a large amplitude of turning. This might arise from central nervous interrelations of the two mechanisms respectively having to do with turning and with creeping, as a kind of reciprocal inhibition; or the relation might arise in a more directly mechanical way. In any case, since something is already known as to the critical thermal increment for the pedal mechanism (Crozier and Pilz, 1923–24), the effect of temperature upon circus movement should tell something as to the nature of the interplay between the activities of the creeping organ and of the parietal elements concerned with the bending of the body. A clear understanding of this matter is essential before one is able to say to what extent the amplitude of an orientation movement due to enforced asymmetrical excitation may legitimately be taken as a measure of the photosensory effect.

The experiments have given a result somewhat unexpected, which, however, seems significant not only for the question immediately concerned, but also for some more general phases of behavior study.

III.

Individual slugs of known size and weight, about 70 animals in all, had either the right or the left eye-tentacle removed. These individuals ranged between 0.3 and 14.0 gm. Beginning several days subsequent to the removal of one eye-tentacle and its eye, they were allowed to creep under a constant vertical illumination. With these conditions, *Limax* tends to circle smoothly and continuously toward the de-eyed side, since the animal is negatively phototropic. The precautions necessary to insure significant results in such experiments are discussed in a succeeding paper (Crozier and Cole). In the present experiments the animal creeps upon a moistened glass plate, 45 cm. in diameter, beneath which is white paper bearing a system of circular and radial coordinates. Creeping is begun (in a straight line) before the light is admitted. The trail pursued while creeping is copied upon a similarly prepared record sheet, scale 1:4; this is done by observing continuously the movement of a morphologically defined point near the apex of the slug's mantle from the moment orientation begins. A trail of about 20 cm., requiring 3 minutes for execution, is usually taken. The exact duration of each observation is obtained with a stop-watch. On the copy of the trail the total length of the path, and the angular displacement of a tangent to the path, are subsequently measured. As the movements of the slug are very slow the record of the trail is obtainable with a satisfactory degree of precision.

The experiments were carried on in a dark chamber. The temperature of the room was controlled by electric heating elements, and by the liberal use of ice. For the lowest temperatures employed the observation stage was surrounded with cracked ice, and the stage (at the bottom of a large glass cylinder) was closed by a glass cover. The temperature was constant within 0.2°C. As source of light a 400 watt concentrated tungsten filament was used, in conjunction with a special glass filter excluding the infra-red but practically non-selective in the visible spectrum. The lamp was mounted in a ventilated "house," the filament in the axis of the observation stage. The stage was surrounded by a white cylinder of bristol board, 20 cm. high. On the creeping platform the photic intensity

was about 135 m.c. The mean deflections obtained at this intensity agree very precisely with the results at corresponding temperatures with a different lot of animals a year previously (by Crozier and Cole).

A small correction for the effective light intensity can be applied by means of measurements of the height of the eye above the creeping platform. This is on the assumption, probably correct but not rigorously demonstrated, that the eye is the essential receptor in the circling response. With the aid of curves giving the relations between the length and weight of individual and the elevation of the eye, the mean elevation was obtained for the animals employed in these tests and this correction (0.7 cm.) was deducted from the measured distance between the incandescent filament and the observation stage.

The slugs were kept in separate glass jars with a little moist decaying wood. They may thus be maintained in an active state for several months, and their individual peculiarities of response studied at leisure. With this side of the matter we are not at present concerned, save to state that the mean results upon which reliance is placed show a very satisfactory degree of consistency.

The data forming the basis of this discussion result from the analysis of 206 trails, not including the large number involved in the previous experiments of Crozier and Cole (1923). From the nature of the record taken, each of these trails itself yields an *average* measure of orientation.

IV.

The average "circus movement effect" is best estimated by the ratio

$$\frac{\text{Degrees total deflection}}{\text{Centimeters length of path}}.$$

This gives a means of numerically expressing the differential bilateral muscle tensions which determine the orienting posture. This measure has particular significance because the posture is continuously maintained during the circling movement (Figs. 4 and 5). There is here no possibility of failure to recognize the effect of light in

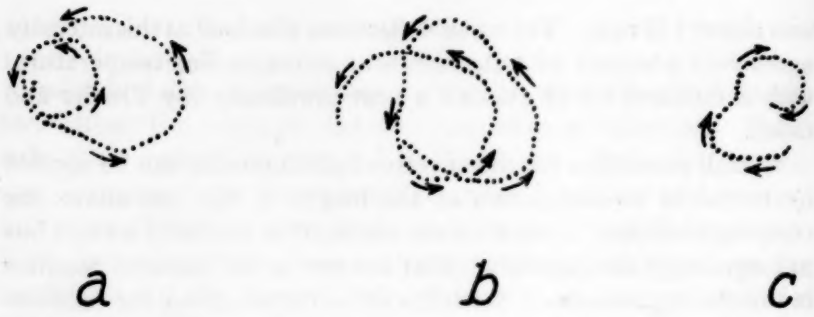

FIG. 4. Typical circus movement trails; *a*, at 21°, 18.73° per cm. deflection, 7.94 cm. per min., speed of creeping; *b*, at 21°, 15.53° per cm., 9.42 cm. per min.; *c*, at 27°, 30.45° per cm., 5.08 cm. per min. Note continuous character of the circling motion. There is no difference, quantitatively, between right-hand and left-hand turns. Scale 1:4.

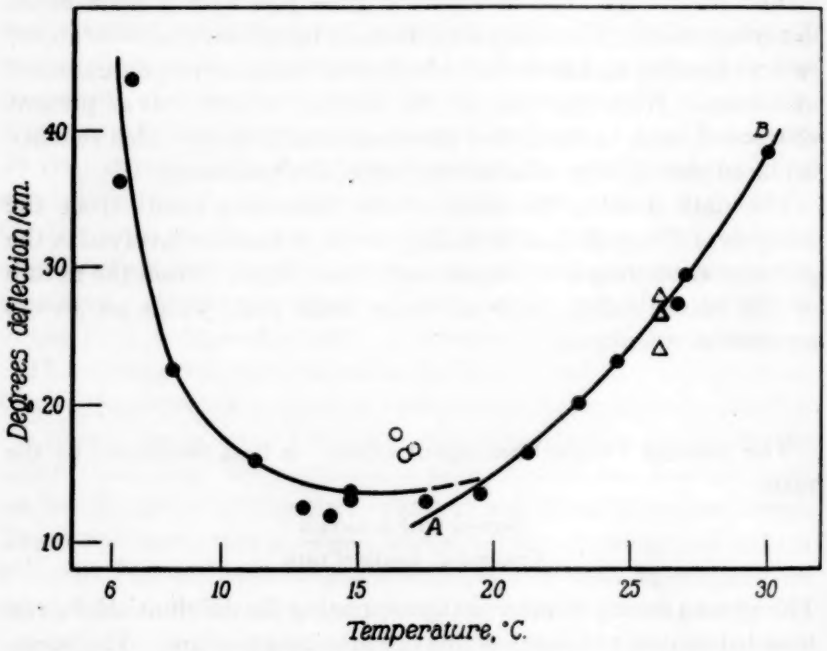

FIG. 5. The ratio *degrees deflection: cm. length of path*, in circus movements under constant vertical illumination, as a function of temperature. Several independent determinations shown as triangles.

producing differential musle tensions. With arthropods the tensions of locomotor muscles on the one hand, and on the other the matter of the rapidity of their phasic contractions, have sometimes been preposterously confused (Mast, 1923–24).

Fig. 5 shows that under constant illumination the expression of the circus movement varies profoundly with the temperature. So also does the speed of creeping (Fig. 6). The amplitude of bending and the speed of progression vary reciprocally. On either

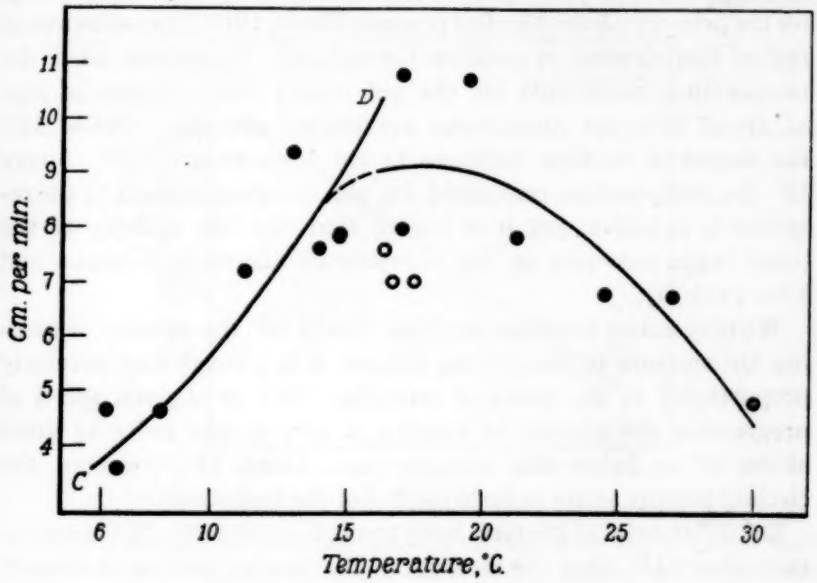

FIG. 6. The rate of creeping during circus movements under constant illumination at different temperatures.

side of an intermediate zone centering at near 15°C., the effect of temperature is reversed. From 15–30° the amount of turning increases as the temperature is elevated; below 15° the degree of turning is enhanced as the temperature falls. The effect on velocity of creeping is exactly the reverse.

This curious situation may perhaps be understood in several ways. The interpretation which follows seems to us at present the most useful and reasonable. It should be remembered that there may be

involved the effects of temperature upon at least (1) the primary photic excitation; (2) the neuromuscular effect of this excitation as expressed in (*a*) the release of the pedal creeping mechanism, and (*b*) in the tendency to turn; (3) the presumptive nervous interrelations between (*a*) and (*b*); (4) the intrinsic activity of the creeping organ, and of (5) the parietal muscles which produce turning.

Inasmuch as the temperature coefficients are high, it is quite unlikely that any influence upon the photochemical processes of excitation is directly implicated. The low temperature coefficient for the primary photochemical process (Hecht, 1919-20, *a*) allows us to regard this element as constant throughout. Moreover, since the temperature coefficients for the net results suffer change of sign at about 15°, the phenomena are clearly complex. Below 15°, the degree of turning increases as the temperature falls. Above 15° the temperature coefficient for the creeping element of the response is negative, yet it is known that the free activity of the pedal organ increases as the temperature increases (Crozier and Pilz, 1923-24).

We may notice first that on either side of 15° the amount of turning, the measure of the circling posture, is in a rough way inversely proportional to the speed of creeping. But at a given speed of progression the amount of turning is very nearly twice as great above 15° as below this temperature. Above 15°, therefore, the circling posture seems to be in control of the final result.

The differential of bilateral body tonus is so related to the temperature, above 15°, that the measure of the turning posture is proportional to the exponential of the reciprocal of the absolute temperature (Fig. 7). This is the relation required by the Arrhenius equation for velocity of chemical reactions (Arrhenius, 1889; *cf.* Crozier, 1924-25). The value of the critical increment is μ = 16,820. The curve *A B* in Fig. 5 is the curve of this equation,

$$\frac{\textit{amplitude of turning at } T_2^\circ}{\textit{amplitude of turning at } T_1^\circ} = e^{\frac{\mu}{2}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)}$$

with μ put = 16,820.

This result is taken to signify that the amplitude of the maintained turning posture is determined (under constant stimulation) by

the velocity of a chemical process. This cannot be a matter simply of intrinsic muscle tonus, for in the pedal muscle fibers, at least, the temperature coefficient for magnitude of contraction is negative (Crozier and Pilz, 1923–24). It depends upon the continuous action of the orienting light. From the nature of the experiment the acting light intensity does not change as the animal moves. If the light be interrupted, the circling response stops almost instantaneously, and the slug either ceases to creep or follows a more or less straight and random course. We may therefore suppose that the sustained

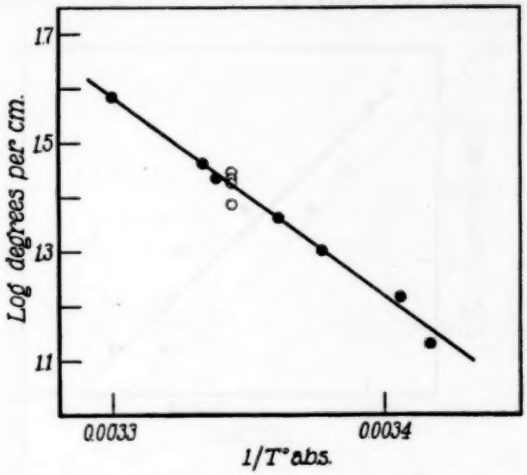

FIG. 7. The logarithm of the amplitude of circling, above 15°, is a linear function of the reciprocal of the absolute temperature. Several independent sets of observations yielded points shown as open circles.

turning posture is due to the continuous output of appropriate nerve impulses from the central ganglia. The magnitude of the critical increment does not agree with that found for some instances of frequency of "nerve center discharge" in arthropods (Crozier, 1924–25), but does agree with the value of μ which may be calculated for several other processes in which oxidations are involved, even when "nerve center" activity may be supposed the governing agency (*cf.* Crozier, 1924–25). The analogy is not a very remote one, because there is indication that in gasteropods the peripheral organs contain nervous structures adequate for autonomous local

behavior, but brought secondarily under the control of impulses from central ganglia (Crozier and Arey, 1919).

The velocities of creeping associated with the turning postures above 15° are in a rough way inversely proportional to the amplitudes of the turning postures. We may assume that at each temperature the animal is creeping as fast as the circling mechanism will permit. At each temperature, then, the reciprocal of the linear creeping velocity might be a measure of the magnitude of the "brake" against which the pedal organ operates. When *Limax* lifts its own weight by creeping vertically upwards, it works constantly against

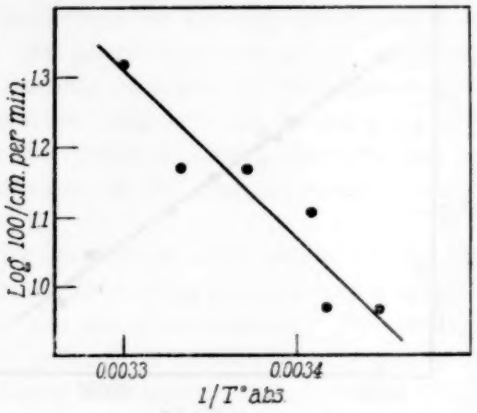

FIG. 8. The reciprocal of the creeping speed (above 15°) as function of temperature; the slope of the line yields μ = approximately 10,900.

a "brake;" the critical increment for pedal activity (frequency of pedal waves) at constant velocity of vertically upward creeping is μ = 10,700, between 11° and 28°C. (Crozier and Pilz, 1923–24).

Fig. 8 shows that the reciprocal of the creeping speed above 15° has a critical increment μ = 10,900, as nearly as can be told. This would seem to indicate that the "slowing down" of creeping is governed by the inability of the intrinsic pedal mechanism to keep pace with the augmented tendency to turn. This agrees with the preliminary assumption made in this discussion, namely that above 15° the tendency to turn is in dominant control of the animal movements. Below 15° the critical increment for velocity

of linear progression is $\mu = 16{,}800$; the curve $C\ D$ in Fig. 6 is the curve of the Arrhenius equation with this value of the constant. The complexity of the creeping act, involving frequency of the pedal waves and their individual efficiency (Crozier and Pilz, 1923–24), is such that a very precise fit cannot be expected. The agreement with the value of the critical increment for amplitude of turning above 15° indicates that the limiting event is of similar type. In this lower temperature range, the amplitude of turning is conceived

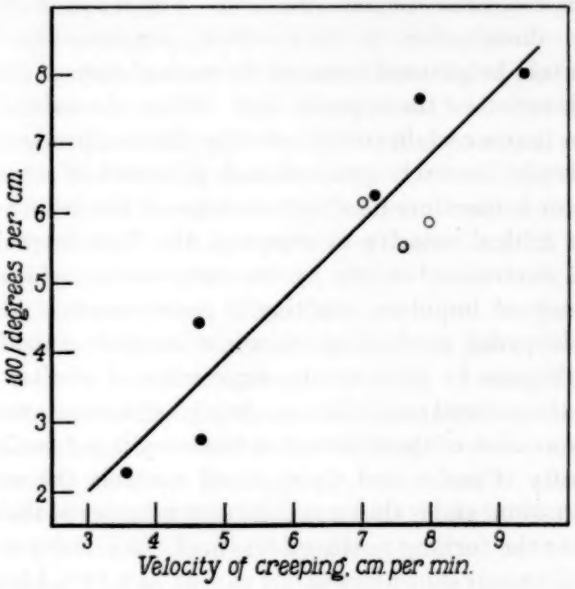

FIG. 9. At temperatures up to about 15° the reciprocal of the amplitude of turning movements is directly proportional to the velocity of creeping.

to be limited merely by the velocity of creeping. The latter is determined by (1) the frequency of impulse discharges initiating pedal waves, and (2) by the temperature. The tendency to turn is being continually excited by the constant action of the light. The fully developed amplitude of bending is therefore limited merely by the speed of progression. As Fig. 9 shows, within this range the reciprocal of the turning amplitude is proportional directly to the associated linear velocity.

V.

The foregoing account makes it appear probable that for the study of circus movement as a function of light intensity, the experiments with *Limax* should properly be made at temperatures well above 15°C. The measurement of the tendency to orient must involve consideration of the relationship between the orientation and creeping. Below 15° the amount of circling is determined very largely by the speed of creeping. The effect of light upon the latter activity is not suitable for measurement. The result of continuous asymmetric illumination is to produce, continuously, impulses which maintain heightened tonus of the musculature of the "blind" side and relaxation on the opposite side. When the animal is creeping with less than a certain critical velocity, the amplitude of bending becomes simply inversely proportional to speed of creeping; its exact amount is therefore no direct measure of the influence of the light. The critical velocity of creeping, the light intensity being constant, is determined chiefly by the temperature, which controls the frequency of impulses resulting in pedal waves. Above 15°, the intrinsic pedal mechanism does not receive central nervous impulses adequate to prevent the expression of similar impulses directed to the parietal musculature. At a given temperature (above 15°) this expression of the orientation tendency is a function of the light intensity (Crozier and Cole), itself controls the velocity of linear progression, and is thus a satisfactory measure of the orienting effect. That the turning posture does check the velocity of progression is very definitely shown by the fact that at, say, 24° a *Limax* known through other tests to be in a phase of complete non-reactiveness to light creeps at a rate three or more times as fast as a similar animal circling normally. The nature of the central interconnections between creeping and turning mechanisms is further illustrated by the fact that a strychninized *Limax* creeps continuously and with high velocity; the drug releases the pedal waves from central inhibitory control.[1] The precision of the relation between temperature and degrees turned per cm. of path shows that this measure of orientation, above 15°, is a delicate index of influences which affect the tonus of muscle groups involved in orientation.

[1] Crozier, W. J., unpublished experiments.

VI.

The relations between temperature and the phototropic circling movements of *Limax* are probably important for a point which is believed to be of some general significance. For the duration of the reflex tonic immobility in the isopod *Cylisticus* (Crozier, 1923–24), it was found that the critical thermal increment below 15°C. differs materially from that obtained above this temperature (Crozier and Federighi, 1923–24). It was pointed out in a preliminary way that this abrupt change in the relation to temperature probably signifies that a different chemical reaction has become the governing process in the series of events determining the duration of the act of immobility. It can be shown how in a catenary system this result may be obtained. This type of alteration in the curve relating temperature to the velocity of vital reactions is not unusual (Crozier, 1924–25). The behavior of *Limax* supplies a gross physical model of the kind of dynamical situation which has been pictured in the central nervous operations of *Cylisticus* and which seems to obtain in a number of other processes. Beginning at a low temperature, the rate of creeping, determined by the activity of the pedal organ, is found to increase as the temperature is elevated. The critical increment is $\mu = 16{,}800$. At about 15° the expression of the velocity of the central nervous activity underlying the rate of discharge from the pedal ganglion becomes about equivalent, dynamically, to that which determines the differential muscle tensions effecting turning. Beyond 15°, the pedal mechanism is in secondary place; the slug then creeps as fast as its tendency to turn will permit—the turning mechanism becomes the controlling element in behavior. At about 15° the "turning impulse" has, so to speak, overtaken the creeping tendency. The difference between this model and a system of interconnected chemical events having diverse temperature characteristics must not be lost sight of. In a catenary system the speed of the slow process determines the magnitude of the total velocity, hence the slowest process has control. In our *Limax* model the phenomenon of greater amplitude, as determined by the temperature, controls the end-result; and when the curves connecting each effect with the temperature "intersect" in the neighborhood of

a particular temperature a profound change becomes apparent in the net result when observed over a range of temperatures on either side of this critical point.

VII.

There are conceivably two ways in which the variability of response in animal conduct may be regarded. Variability (unpredictability) may be taken as a category, and used as the foundation of a theory of behavior; or one may attempt to analyze it. The former is that method sometimes referred to as anthropomorphism. It has not led to useful results. The analysis of variability of response has scarcely begun. It may be assisted in an important respect by the consideration of such instances as the one we have discussed.

The essence of the situation is this: experiment with *Limax* over a range of temperatures shows that in the execution of circus movements under light of constant intensity two chief factors are involved—the rate of creeping, and the amplitude of turning. The structural basis for each element of the response is distinct, but the two are interrelated in various ways. Within a certain zone of temperatures in the neighborhood of 15° and 16°C., which probably would be commonly taken as "normal" for this animal, the response is distinctly more variable, quantitatively, than at temperatures either higher or lower. In this zone of "normal" temperature conditions, the dynamical balance between the tendency to creep and the tendency to turn is close to a critical point. Slight circumstances may determine in particular instances which of these two tendencies is dominant. Hence the relatively great variation in individual results. At higher temperatures the turning mechanism is in control, and the animal creeps only so fast as the inner mechanism of turning will permit. At lower temperatures the amount of turning is limited merely by the speed of creeping.

It follows that under natural environmental conditions certain types of variability in response may be conceived to result from the fact of delicate dynamic equilibrium among processes which (in effect) compete for control of the animal's mechanisms of movement. And the especially curious principle ensues, that for the real understanding of the elements determining animal conduct it is almost

essential, therefore, to place the organism under distinctly "abnormal" conditions such as are favorable to the expression now of this, now of another element in the normal nexus.

VIII.

SUMMARY.

1. The theory of animal phototropism requires for particular instances a knowledge of the action of light as exerted through each of two bilaterally located receptors functioning singly. The measurement of "circus movements" which this involves must be concerned with such aspects of the reaction as are demonstrably dependent upon the effect of light.

2. The negatively phototropic slug *Limax maximus* exhibits very definite and continuous circus movement under vertical illumination when one eye-tentacle has been removed. The amplitude of the circling movement, measured in degrees deflection per cm. of path as an index of maintained differential tonus, is intimately related to the concurrent velocity of creeping. Analysis of the orienting mechanism is facilitated by the fact that in gasteropods such as *Limax* the animal creeps by means of the pedal organ, but orients (turns) by a totally distinct set of muscles in the dorsal and lateral regions of the body wall.

3. The expression of the phototropic orienting tendency, with illumination constant, is greatly influenced by the temperature. Above a zone centering at 15°, the amplitude of turning (degrees per cm. of path) is determined by the temperature in accurate agreement with Arrhenius' equation for chemical reaction velocity, with the critical increment $\mu = 16{,}820$; and the rate of creeping is progressively less as the temperature rises, μ for its reciprocal being 10,900. Below 15°, the velocity of creeping becomes less the the lower the temperature, μ being again 16,800; while the amplitude of orientation is limited merely by the velocity of creeping, its reciprocal being directly proportional thereto.

4. Measurements of *Limax* circus movements in terms of turning deflection as function of light intensity must therefore be carried out at a temperature well above 15°.

5. The analysis provides a gross physical model of how an end-result may be influenced by temperature according to the effect of temperature upon each of several interconnected processes when the "temperature *vs.* effect" curves for these processes dynamically intersect.

6. It is pointed out that a certain type of unpredictability (quantative variability) in animal behavior under "normal" natural conditions probably results from dynamic equilibrium there obtaining between diverse mechanisms competing for effector control (in the present case, the creeping mechanism and that for turning, in the range 14–46°C.). It follows that the unraveling of the elements of conduct necessitates experimentation under diverse abnormal conditions favoring individual mechanism of response.

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THE INFLUENCE OF OXYGEN TENSION UPON METABOLIC RATE IN INVERTEBRATES.

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(Accepted for publication, August 14, 1924.)

Since the work of Thunberg (1) on air breathing invertebrates (*Lumbricus*, *Limax*, *Tenebrio*) it has been recognized that the oxygen consumption of some of the lower animals is at least partially dependent upon the oxygen tension in the surrounding air or water. Henze (2) reports that the metabolic rate of some of the simpler marine invertebrates (*Actinia*, *Anemonia*, *Sipunculus*) shows a dependence upon the oxygen tension in the water, decreasing as it decreases; whereas the metabolism of high invertebrates (*Carcinus*, *Scyllarus*, *Aplysia*, *Eledone*) is independent of the oxygen tension over a considerable range. McClendon (3) finds that the oxygen consumption of *Cassiopea* diminishes as the tension of the oxygen in the water is reduced. Lund (4) reports that the oxygen tension becomes a limiting factor in the oxygen consumption of *Planaria* when the tension is reduced below one-third saturation of the water, but finds (5) that *Paramecium* is independent of the oxygen tension over a wide range. Krogh (6) accepts Henze's observations and interpretation and arrives with him at the general conclusion that the metabolic rate of cold-blooded animals is independent of the oxygen tension in the surrounding medium except in some of the simpler organisms, where, because of inadequate respiratory or circulatory mechanisms, the oxygen tension in some of the tissues becomes zero. In such forms the rate of diffusion of oxygen into the tissues must vary with the oxygen tension in the environment, and the metabolic rate must be similarly affected. Krogh believes that Thunberg's results are to be explained in this manner, the animals which he investigated having somewhat imperfect respiratory mechanisms.

We have secured evidence, which indicates clearly that Henze's distinction between the simpler and the more complex invertebrates is by no means absolute. We are able to show that the metabolic rate in some of the higher marine invertebrates, with well developed respiratory mechanisms,is closely dependent upon the oxygen tension in the water over a wide range. We have conducted the experiments in such a way that the data assume a dynamic form, the experimental values giving a consistent series, adapted to mathematical treatment and related by a simple law.

For the determination of dissolved oxygen we have used Birge and Juday's modification (7) of the Winkler method. We have followed the rate at which various animals remove oxygen from a quantity of sea water, the reduction of oxygen tension being effected by the metabolism of the animals themselves. We have conducted the experiments in glass aquaria of various sizes, in glass bottles, and in other similar vessels. Determinations of the oxygen content of the water are made at various times during the course of the experiment, test samples being withdrawn through a siphon into small test-bottles of about 35 cc. capacity. The loss in volume due to the removal of water for testing is thus reduced to the minimum consistent with accurate analysis. In many experiments we have replaced the water withdrawn by a like amount of water made up to an oxygen tension identical with that indicated by the preceding analysis. Constant volume is thus maintained. In no case has the amount withdrawn constituted more than 5 per cent of the total quantity of water used. In all cases the water surface has been covered with a thick layer of paraffin oil, to retard the diffusion of oxygen into the water from the air. Suitable control tests have shown that under our experimental conditions the diffusion of new oxygen into the water from the air is negligible in amount.

Using this simple technique we find that when the oxygen tension diminishes slowly, the marine fish, *Fundulus heteroclitus*, is able to remove equal amounts of oxygen in equal times down to about 10 per cent oxygen saturation, showing thereby that its metabolism is independent of the oxygen tension over a wide range, as Winterstein (8) previously found for fresh water fishes. With more rapid rates of reduction a slight dependence of metabolic rate upon oxygen

tension may become apparent. In the metabolic regulation respiratory and other reflexes are apparently involved.

In striking contrast with the relative independence of these vertebrate forms we find that the oxygen consumption of the lobster,

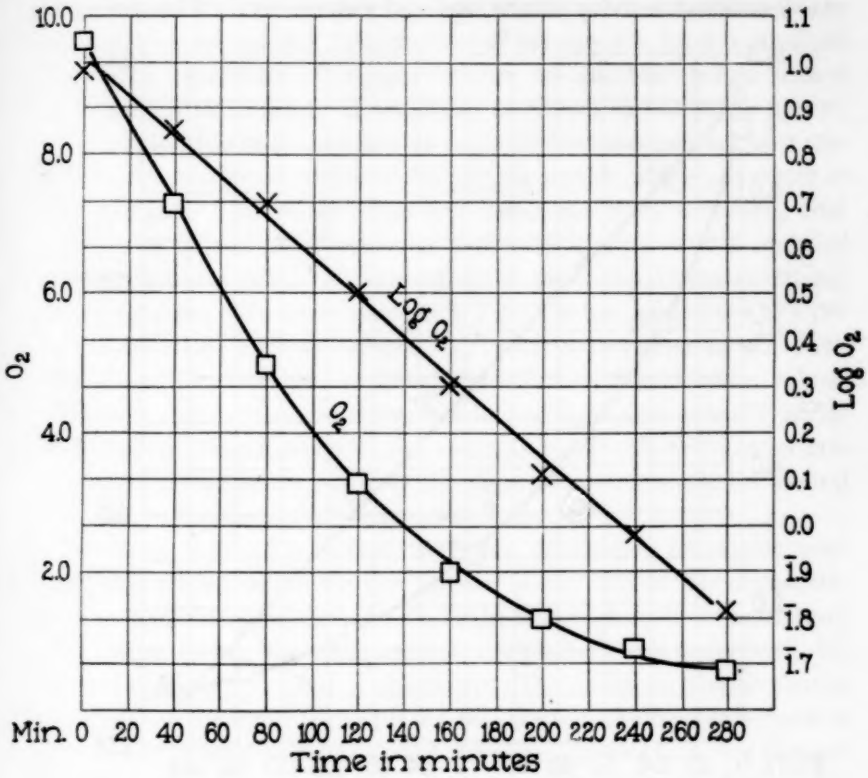

FIG. 1. Rate of oxygen consumption by *Homarus americanus*. Squares represent oxygen tensions remaining in the water plotted against time; crosses represent the logarithms of these tensions plotted against time. Oxygen values are expressed in cc. of N/300 thiosulfate. Capacity of test-bottle = 35 cc., 1 animal. Temperature = 20°C.

Homarus americanus, and of the annelid worm, *Nereis virens*, is quite dependent on the oxygen tension in the water. The lobster possesses respiratory and circulatory mechanisms whose efficiency compares favorably with those of aquatic vertebrates. Those of

Nereis are less well developed. In the lobster experiments single animals have been used; in the *Nereis* studies groups of from 25 to 100 worms. The lobsters stir the water of the aquaria very efficiently by means of the constant movements of the respiratory apparatus, and the occasional activity of the legs and swimmerets. The worms are

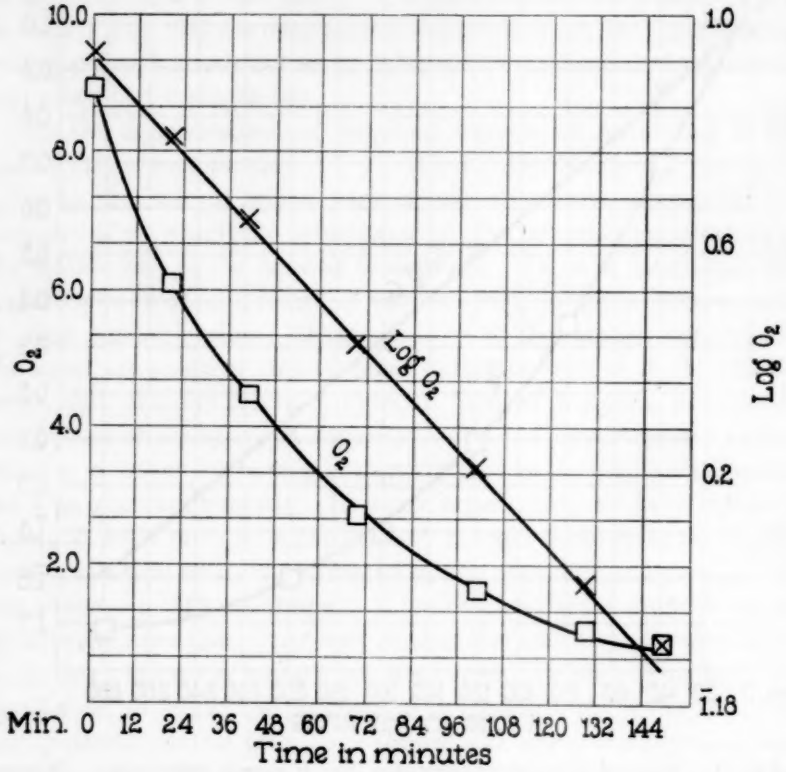

FIG. 2. Rate of oxygen consumption by *Nereis virens*. Squares represent oxygen tensions remaining in the water plotted against time; crosses represent logarithms of these tensions plotted against time. Oxygen values are expressed in cc. of N/300 thiosulfate. Capacity of test-bottle = 35 cc., 82 animals. Temperature = 20°C.

relatively sluggish, and in all experiments the water has been continually mixed by a motor-driven stirrer projecting into the water through the oil seal.

For both animals the experimental values assume the same form. Fig. 1 shows a typical curve of oxygen consumption given by *Homarus;* Fig. 2 a similar curve given by *Nereis.* In both it is seen that as the oxygen tension is reduced the animals consume smaller and smaller absolute amounts of oxygen, so that when oxygen remaining in the water is plotted against time, a curve of exponential form is obtained When the logarithms of oxygen tension are plotted against time a straight line relationship is obtained, as shown in the same figures. This relationship indicates that at every instant the oxygen consumption is directly proportional to the oxygen tension in the sea water at that instant. The relationship has come out with surprising consistency in dozens of experiments and holds closely over a range of oxygen tensions from full saturation of the water down to 10 per cent saturation. In some cases, as in Fig. 2, it has held down to 7 per cent saturation, and we believe that with refinements in the technique it will be possisble to show that it holds for even lower values. Moreover, the curves are reproducible and the same animals, under identical experimental conditions, again and again show the same rate of oxygen consumption, so that the determinations made for several successive experiments fall upon a single curve.

We have satisfied ourselves that this diminution in oxygen consumption with lowered oxygen tension is not due to the accumulation and inhibitory action of CO_2 in the water, since identical curves are obtained when the same animals are placed in sea water with different CO_2 tensions. The production of CO_2 does cause a slight change in the pH of the water (from pH 8.1 to 7.6 when the oxygen tension is reduced to 10 per cent saturation of the water) but we find that changes in CO_2 tensions much exceeding this amount have no effect upon the rate of oxygen consumption.

The mechanism of the observed effects is not clear. The evidence suggests that some simple physical process, such as the diffusion of oxygen into tissues where the oxygen tension is zero, is the controlling factor. Our data accord exactly with the theoretical expectation for such a diffusion process, since the amount of oxygen delivered by diffusion into such tissues would always be proportional to the external tension. The complexity of the organisms, however, renders impossible any final judgement as to the mechanism of the observed

effects. We hope to push the analysis further, and are continuing the study.

In a few preliminary experiments we find evidence that the oxygen consumption of the king crab, *Limulus polyphemus*, and of the blue crab, *Callinectes sapidus*, is also directly dependent upon the oxygen tension of the water. On the other hand the shrimp, *Palæmonetes vulgaris*, appears to be able to regulate its metabolism down to about 50 per cent oxygen saturation of the water, extracting equal amounts of oxygen in equal times. At lower oxygen tensions its curve of oxygen consumption swings over to the exponential form, similar to that of *Homarus*. The squid, *Loligo pealei*, shows even better regulation, down to about 30 per cent.

It would appear, then, that no general law can be stated for the higher invertebrates; but it is clear that some of them are quite dependent upon the oxygen tension of the surrounding medium, and it is suggested that this dependence is a factor of importance which should be recognized in all studies in which measurements of oxygen consumption are being made. Many workers have assumed that oxygen consumption is independent of oxygen tension, and by the determination of two values, at the beginning and at the end of the experiment, have made calculations of the oxygen intake per unit of weight and time. For all animals in which the oxygen consumption is controlled by the external oxygen tension, such measurements will obviously give incorrect values.

SUMMARY.

It is shown that in several of the higher invertebrate animals, oxygen consumption is directly proportional to the oxygen tension in the sea water, over a wide range.

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